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SUSCEPTIBILITY AND RESISTANCE OF *ANOPHELES QUADRIMACULATUS* TO *DIROFILARIA UNIFORMIS*

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Recently Price (1957) described a filariid nematode from the subcutaneous connective tissue of a cottontail rabbit and named it *Dirofilaria uniformis*. The life cycle of this parasite was elucidated by Bray and Walton (1960) who found that the microfilariae developed in the hemocoele of *Anopheles quadrimaculatus*. Further investigations, Moon et al (1960) revealed that Dutch breed domestic rabbits may be suitable for routine maintenance of the infection in the laboratory.

In order to determine whether *D. uniformis* could be utilized conveniently for laboratory studies of filarial infections, it was considered essential to know more about the mosquito phase of its life cycle. This report deals with some of the factors which influence the development of *D. uniformis* in the invertebrate host.

MATERIALS AND METHODS

The life cycle of *D. uniformis* has been maintained for several years in this laboratory by using the wild rabbit *Sylvilagus floridanus mallurus* and the Dutch breed domestic rabbit as the vertebrate hosts. *A. quadrimaculatus* mosquitoes obtained from the Entomological Research Division, U.S. Dept. of Agriculture, Orlando, Florida, served as the intermediate hosts.

All mosquitoes used in these experiments were maintained routinely on 4 percent sucrose solutions and apples. They were allowed to feed on rabbits either by inserting the animals' heads into the mosquito cage or by placing the mesh-covered surface of a small plastic cylinder with a given number of mosquitoes against the shaved abdomens of the rabbits. Several small containers were used when simultaneous feeding of different age or infection groups was necessary.

Infected mosquitoes were dissected in 0.35 percent saline under a stereoscopic microscope .The filarial larvae were counted and a record was made of their location and morphology. When measurements were required, larvae were placed in 2 percent formalin and the length determined with the aid of a calibrated ocular micrometer of a compound microscope. In some studies, the mosquitoes were kept in cabinets where constant temperatures and a relative humidity of 75 to 85 percent were maintained.

RESULTS

The first experiment was designed to determine the effect of single infections of varying sizes on the survival of mosquitoes and on the numbers of developing larvae. *A. quadrimaculatus* adults were fed on rabbits with different degrees of microfilaremia (table I). The percentage of anophelines surviving and the length of survival decreased with increasing intensity of the microfilaremia. Within the experimental limits increases in the size of the infective dose brought about a decrease in the percentage development of larvae. Therefore, the mean number of larvae developing was similar in the first three groups of mosquitoes. However, the few surviving mosquitoes of the fourth group which received an overwhelming infection harbored a much higher number of larvae.

TABLE I. *Relationship between microfilaremia and development of infective larvae in* Anopheles quadrimaculatus.

Microfilaremia (Mf/0.02 ml. blood)	Number of mosquitoes fed	Mosquitoes surviving 10 days		Mean survival (days) exposure until death	Mean no. of larvae
		Number	Percent		
95	39	25	64	6.6	23
253	37	18*	49	5.4	21
310	53	23	43	5.8	22
3395	120	5	4	< 1.0	74

* Including 1 uninfected mosquito.

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Preliminary observations on the distribution of infective larvae in the mosquitoes indicated that approximately one-half of them were recovered from the head and proboscis, the rest being distributed between the thorax and the abdomen. A second experiment was designed to determine whether the intensity of infection of mosquitoes affects the distribution of larvae. The results of this experiment, involving 757 mosquitoes from which more than 15,000 larvae were recovered, indicate that the distribution of larvae is essentially alike regardless of the size of infection (table II).

TABLE II. *Relationship between intensity of infection and distribution of infective larvae in* Anopheles quadrimaculatus.

Infective larvae/ mosquito	Number of mosquitoes dissected	Total no. larvae	Distribution of larvae 10 days after infection					
			Head		Thorax		Abdomen	
			No.	Pct.	No.	Pct.	No.	Pct.
1– 5	76	202	101	50	28	14	73	36
6–15	142	1,673	1,045	62	322	19	306	18
16–35	448	10,281	5,943	58	1,918	19	2,420	24
>35	91	3,541	2,014	57	658	19	869	25

In order to test whether larvae from different regions in the mosquitoes are equally infective, six rabbits were separated into three groups and injected subcutaneously with known numbers of infective larvae from the head, thorax, and abdomen, respectively. No significant differences in the percentage of development occurred. In the light of these preliminary results, another experiment was set up to study whether the loss of infective larvae from the mosquitoes during feeding occurs from the head and proboscis only. Mosquitoes were fed on an infected rabbit and after 10 days half were allowed to feed on a normal rabbit while the others were kept as controls. At this time, all mosquitoes were sacrificed and the number of larvae recovered from the head, thorax, and abdomen was counted. Re-feeding produced a loss of 58 percent of the larvae harbored by the mosquitoes (table III). Although the loss was greatest from the head and the thorax, there was also some reduction in the number of larvae from the abdomen.

TABLE III. *Effect of re-feeding after 10 days on distribution and loss of larvae.*

Exper. group	Number of mosquitoes	Number of larvae recovered	Distribution of larvae									Percentage loss by refeeding		
			Head			Thorax			Abdomen			Head	Thorax	Abdomen
			No.	Pct.	S.D.	No.	Pct.	S.D.	No.	Pct.	S.D.			
I Refed	30	634	366	58	14.8	44	7	1.5	224	35	6.2	65	75	20
II Controls	30	1509	1055	70	21.0	173	11	5.8	281	19	7.6	—	—	—

In an attempt to determine the role of temperature in the development of *D. uniformis* larvae, mosquitoes were maintained at 23, 27, and 31 C after feeding on infected rabbits. Eleven mosquitoes from each of the three groups were dissected 4, 7, 10, and 13 days after infection. The number of larvae recovered from mosquitoes maintained at 27 C was considerably higher than in the groups kept at 23 and 31 C (figure 1). The larvae developing in the mosquitoes maintained at 31 C grew more rapidly during the first 7 days than those at the two lower temperatures, but by the 10th day the larvae at 27 C had attained the greatest mean length (figure 2).

In order to determine the effect, if any, of the mosquito's age on the number and growth of developing infective larvae, several groups of *A. quadrimaculatus* varying in age from 4 to 13 days after emergence from the pupal stage were exposed to infection at the same time and dissected 3, 6, and 9 days later. A substantial difference was observed in the number and length of larvae recovered in the various groups (table IV). Significantly more and longer larvae were recovered on the sixth and ninth days from mosquitoes 12 to 13 days old than from those 4 to 5 days old. These differences are highly significant with a P of less than 0.01.

Two experiments were set up in order to study the effect that a primary infection might have on the number and length of larvae developing from a superinfection. In these experiments, controls of first and second infections were used to learn what effect, if any, the second infection might have on the larvae of the previous infection. In both experiments

TABLE IV. *Effect of Mosquitoes' Age on Development of Larvae.*

Age of mosquito when infected (days)	Larvae recovered from five mosquitoes					
	3 days		6 days		9 days	
	Number	Mean length microns	Number	Mean length microns	Number	Mean length microns
4–5	99	259	22	503	85	932
9–10	41	247	75	645	134	991
12–13	155	249	128	749	155	1048

TABLE V. *Effect of reinfection of mosquitoes on the number and length of larvae.*

	Type of blood meal		Day of mosquito dissection	Number of larvae recovered from five mosquitoes			Mean length of larvae (microns)		
	Day 0	Day 10		1st inf.	2nd inf.	Prob.	1st inf.	2nd inf.	Prob.
1st Experiment	Infected	Infected	14	82	121	$P < 0.001$	1127	275	$P < 0.001$
	Normal	Infected	14	—	178		—	250	
	Infected	Normal	14	50	—		1083	—	
	Infected	Infected	17	35	169	$P = 0.08$	1172	761	$P > 0.01$
	Normal	Infected	17	—	203		—	746	
	Infected	Normal	17	98	—		1176	—	
2nd Experiment	Infected	Infected	14	84	19	$P < 0.001$	1220	250	$P < 0.001$
	Normal	Infected	14	—	48		—	283	
	Infected	Normal	14	74	—		1223	—	
	Infected	Infected	17	66	37	$P = 0.14$	1193	833	$P = 0.05$
	Normal	Infected	17	—	51		—	900	
	Infected	Normal	17	57	—		1164	—	

fewer larvae from the second feeding developed in mosquitoes which had been previously infected (table V). The difference between the number of infective larvae recovered in the superinfected mosquitoes in relation to the controls is significant at 14 days with a P value of less than 0.01. Although the results of the one experiment suggested the possibility that the first exposure might bring about a reduction in the normal growth of larvae of the challenging exposure, the results were reversed in the other experiment. No significant difference was found in the number and size of the larvae of the primary infection in the reinfected mosquitoes and in their control.

DISCUSSION

In the first experiment, an increase in the infective dose up to a certain threshold brought about a corresponding decrease in the percent

FIGURE 1

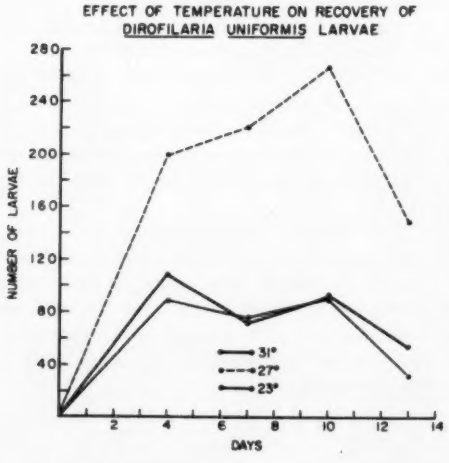
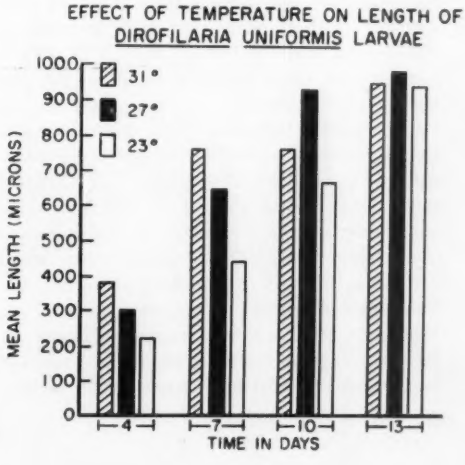


FIGURE 2



recovery of larvae. Beyond that, the parasites produced rapid death of nearly all the infected mosquitoes. In view of these findings, a microfilaremia of approximately 100 per 0.02 ml of blood appeared to be the optimal dose to produce infective larvae in *Anopheles quadrimaculatus* in sufficient numbers to insure routine transmission of the infection. The fact that a relatively large number of infective larvae developed in those few mosquitoes which survived an overwhelming dose would indicate that the results of the other groups with lower infections were not due to physiological crowding of the larvae, but possibly either to an increased host response to a greater stimulus or to the fact that only those anophelines with lighter infections survived. This phenomenon has been observed in vertebrates infected with varying doses of nematodes (McCoy, 1931; Sadun, 1949).

The recovery of infective larvae in the thorax and the abdomen of mosquitoes as well as in the head is of interest and of greater significance in the light of the subsequent findings that there is a loss of larvae at feeding from each of the three regions in the body of the mosquito. The latter observation is in agreement with results obtained by Jordan (1959) with *Wuchereria bancrofti* in infected *Culex fatigans*.

A direct relationship was observed between the age of mosquitoes at infection and the number and length of the larvae developing from them. This is in contrast to the report that *Aedes aegypti* became more resistant with age to infection with the malarial parasite *Plasmodium gallinaceum* (Terzian et al, 1956). However, attention should be called to the fact that in our experiments increased susceptibility may be related to senility. The relation of age of insects to resistance is influenced by many factors among which of greatest importance are the intrinsic qualities of the insects' tissues or metabolism on the physiology of the parasites (Steinhaus, 1949). These observations which are of interest in the understanding of the biological interrelationships between the invertebrate hosts and their parasites should not be taken to suggest the use of older mosquitoes in the routine laboratory transmission of this infection. On the contrary, the mortality among older *A. quadrimaculatus* was so much greater as to make this procedure impractical.

Of considerable interest was the suggestion of an acquired resistance in *A. quadrimaculatus* toward superinfection with *D. uniformis*. Naturally acquired immunity has been found present in insects in nature by d'Herelle (1911) who reported that nearly one-fourth of the grasshoppers in epizootic areas had acquired an immunity to *Cocobacillus acridiorum*. Since then, numerous instances have been reported in which active immunity has been produced in insects using a variety of microorganisms, mostly bacteria. Although both humoral and cellular factors are considered important in the immunity process of an insect, just which tissues, systems, or substances of the insect's body determine the nature of intensity of the humoral or cellular immunity has not been thoroughly established (Steinhaus, 1949). The relatively large size of helminth larvae and the fact that they do not multiply in the mosquito's body, thus providing an instrument for quantitating the degree of resistance, should make them ideally suited for further immunological studies.

SUMMARY

Anopheles quadrimaculatus adults were fed on rabbits infected with *Dirofilaria uniformis* with varying degrees of microfilaremia. Increases in the size of the infective dose, up to 310 microfilariae per 0.02 ml of blood, brought about a decrease in the percentage development of the larvae resulting therefrom.

Approximately one-half of the infective larvae were recovered from the head and proboscis, the rest being distributed between the thorax and abdomen. There was a loss of larvae from each of the body regions of infected mosquitoes taking a second blood meal. Larvae from each of these regions were infective for rabbits.

The optimum temperature for the survival and growth of larvae during their development was 27 C. Significantly, more numerous and longer larvae developed in older mosquitoes. Some evidence of resistance to superinfection was observed in mosquitoes and its implication was discussed.

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RESEARCH NOTE

INTRAVENOUS INOCULATION OF THIRD-STAGE LARVAE OF *METASTRONGYLUS* SPP. AND IMPLANTATION DIRECTLY INTO SWINE LUNGS.

It has been demonstrated that *Dictyocaulus viviparus* find their way into the lungs of calves after subcutaneous injection into the cervical, flank and gluteal regions. (Wade and Swanson, 1958, Am. J. Vet. Res. 19: 792–793). *Metastrongylus* spp likewise establish in swine lungs after injection of third stage larvae into the blood stream or implantation directly into the bronchioles.

Two six-week-old pigs which had not been exposed previously to lungworms were given 3d-stage larvae of *Metastrongylus* spp. One pig was administered approximately 2000 larvae via the anterior vena cava. About 2000 infective larvae were introduced into the bronchi of the second pig via a tube which was inserted through an incision in the trachea. The pigs were maintained in a concrete-floored room to preclude their exposure to earthworm immediate hosts.

Numerous adult lungworms were found in each of the pigs at necrospy, 27 days following inoculation. This brief experiment demonstrates that 3d-stage larvae of *Metastrongylus* spp. do not require action of gastric juice or intestinal secretions as a prerequisite of parasitism.—GEORGE W. KELLEY, JR., *University of Nebraska*, and DOUGLAS KROUS, *Veterinary College, Kansas State University*. (Published with the approval of the Director of the Nebraska Agriculture Experiment Station as paper No. 1068.)

RESEARCH NOTE

A RECORD OF *BORRELIA* FROM THE BLACK-THROATED HIMALAYAN JAY, *GARRULUS LANCEOLATUS* VIGORS, 1832: A POSTHUMOUS RECORD.

During the course of an epizootiological survey on toxoplasmosis, peripheral blood smears of 101 wild birds belonging to different species that were trapped in the reserve forest area around the Indian Veterinary Research Institute, Mukteswar (Kumaon hills, Uttar Pradesh), were examined for determining the prevalence of different haematozoa. Accidentally, a spirochaete was observed in films obtained from a female specimen of a black-throated Himalayan jay, *Garrulus lanceolatus* Vigors, 1831. When examined under dark-field illumination, wet mounts of drawn blood revealed several spiral cells showing active motility with lashing movements. The architecture of coils could be better appreciated when unstained films were studied under a phase-contrast microscope. The ends appeared pointed and the coils numbered, on an average, two to three. The double-contoured appearance exhibited by the jay strain under dark-ground microscopy enabled one to put it in the tick-borne group (Baltazard, 1946, Bull. Soc. Path. Exot. **40**: 77–82). Infected citrated blood was found to contain viable spirochaetes even after storing it at 0 C for 3 but not 4 weeks.

The coarse spirals stained easily with ordinary aniline dyes and violet with Giemsa's stain. In Romanowsky-stained smears, the cells measured, on an average, 8 microns in length and about 0.4 micron in width, with 2 to 3 shallow irregular spirals tapering to a fine filament. Thus, this spirochaete was found to be shorter, thicker, and with less number of turns than *B. anserina*. The ends were pointed and the looser coils with the absence of semicircular terminal hooks differentiated it from *Leptospira*. They reacted negatively to Gram's stain; Tunnicliff's modification brought out the spirochaetes clearly. The organism was soluble in 10 percent ox bile and 10 percent saponin. This strain differed from *Treponema* in having loose rather than tight coils and in the ease with which it could be stained. Growth occurred in Noguchi's ascitic fluid-rabbit kidney medium or Galloway's coagulated egg-white rabbit serum medium, either under aerobic or under anaerobic conditions. But the strain could not be maintained serially on Galloway's medium and was lost abruptly after the 10th subculture. Ticks recovered from infected birds were identified as *Argas miniatus* which species is one of the known vectors of avian spirochaetosis caused by *B. anserina*. However, no transmission could be achieved when these ticks were allowed to test-feed on healthy jays. It was found to be nonpathogenic for mice, guinea-pigs, rabbits, and rats, as well as chicks.

In naturally infected jays, there was no tangle formation at crisis as in the case of *B. anserina*, but the spirochaetes seemed to distintegrate and disappear. The possibility that it could have been a biotype of *B. anserina* was precluded by the fact that there was found to be no cross-immunity between the new species of *Borrelia* and *B. anserina*, as chicks that were inoculated with infected blood containing the jay strain succumbed to a challenge carried out 3 weeks later with *B. anserina*.

Biologically, it may be of importance to note that, besides the black-throated jay, other members of the family *Corvidae* like the Indian crow (*Corvus splendens splendens*, Vieill., 1817) have been shown to be susceptible to artificial infection with *B. anserina* (Knowles, Das Gupta and Basu, 1932, Indian Med. Res. Mem. **22**: 1–113). We could infect healthy jays with this strain but, unfortunately, the jay strain failed to infect chicks, crows and pigeons.

The jay strain consisted of flexuous spiral forms with at least two complete turns, and had no definite protoplasmic structure; thus, it comes under the family *Treponemataceae*. The fact that the jay strain stained easily with ordinary aniline dyes placed it in the genus *Borrelia* of the family *Treponemataceae*.

The speciation in this genus is based on zoological source of origin and, interestingly, only one species of *B. anserina* Sakharoff, 1891 (Bergey *et al.*, 1925), from birds has been recognized so far. It should be emphasized that the jay strain resembled to a great extent the spirochaetes described by Harris (1930, Am. J. Hyg. **12**: 537–568) from caeca of chicks and by Steinhaus and Hughes (1947, Pub. Hlth. Rept. **62**: 309–311) from eggs infected with chicken liver tissue. The points of resemblance were smaller size and more tight coils as well as nonpathogenicity for chickens, guinea-pigs, and mice. The absence of cross-immunity between the jay strain and *B. anserina* coupled with differences in density of growth in laboratory media and morphological differences in the number and amplitude of spirals indicated that the spirochaete described above is possibly a new species of the genus *Borrelia*.—P. G. PANDE, R. R. SHUKLA AND P. C. SEKARIAH, *Toxoplasma Laboratory, Indian Veterinary Research Institute, Mukteswar-Kumaon, U. P., India.*

MAINTENANCE OF *DIROFILARIA UNIFORMIS* IN LABORATORY ANIMALS*

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In 1957 Price described *Dirofilaria uniformis* from the subcutaneous tissue of cottontail rabbits. Bray and Walton (1961) studied the development of the infection in the cottontail and laboratory rabbits and reported that microfilariae develop readily in *Anopheles quadrimaculatus* mosquitoes. Preliminary investigations of the above authors confirmed by the present investigators indicated that only moderate infections of short duration occurred in New Zealand white rabbits.

In the present study attempts were made to establish and maintain the infection in laboratory animals in the hope that this parasite could be used for chemotherapeutic and immunologic investigations.

MATERIALS AND METHODS

Cottontail rabbits (*Sylvilagus floridanus mallurus*) which had been infected with *D. uniformis* in the laboratory by Bray and Walton (1961) were used initially as a source of microfilariae. Additional wild rabbits were trapped at the Patuxent Wildlife Research Center, Laurel, Maryland and experimentally infected in the laboratory.

A strain of *A. quadrimaculatus* mosquitoes was obtained from the Entomological Research Division, U. S. Department of Agriculture, Orlando, Florida, and kept in plastic cages covered with moist cotton. Mosquitoes (*Anopheles quadrimaculatus*) were infected by placing them in containers with a mesh bottom through which they were fed on the shaved abdomens of rabbits with microfilariae counts of 100 to 300 per 0.02 ml of blood. After 10 days mosquitoes were dissected and infective larvae were removed and suspended in 0.35 percent saline solution. Preliminary experiments had shown that larvae from the mosquitoes' thorax and abdomen as well as from the head could infect rabbits and develop into adult worms. Therefore, infective larvae from all three regions were injected subcutaneously into test animals.

The microfilarial counts were made on 0.02 ml smears of peripheral blood which had been laked with water. Knott's (1930) concentration technique was used to detect light infections.

At necropsy the animals were skinned and worms were removed from the subcutaneous connective tissue, counted, and placed in 0.9 percent saline in a refrigerator at 4 C. The following day they were measured on a millimeter scale.

Besides rabbits, young adult guinea pigs, hamsters, rats, mice and suckling mice were used.

RESULTS

Five guinea pigs, four hamsters, three rats and four mice received up to 125 larvae each and were necropsied between 34 and 170 days after inoculation. No filariae were recovered from any of them. Sixteen suckling mice were inoculated with infective larvae and necropsied at intervals from 3 to 139 days later. Larvae were recovered after 3, 5, and 7 days, but they showed no evidence of development; neither larvae nor adult worms were recovered from mice sacrificed subsequently.

Young adult and suckling rabbits of New Zealand white and mixed breeds failed to develop sufficiently high microfilaremias (more then 50 microfilariae per 0.02 ml) for practical maintenance of the infection in the laboratory (table I). Microfilaremias were of short duration and, in general, necropsies performed more than 200 days after infection yielded only disintegrating worms encapsulated by the host tissues. The suckling rabbits were less resistant as judged by relatively higher and longer lasting microfilaremias.

In three rabbits, an attempt to interfere with the host response by injections with 15 mg cortisone, repeated after 48 hours, was unsuccessful.

D. uniformis larvae developed into mature worms and produced microfilariae in suckling New Zealand rabbits with a prepatent period similar to that observed in cottontail rabbits. However, the number of microfilariae in the peripheral blood was usually much lower than in wild rabbits at a comparable stage of infection.

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* The encouragement and valuable suggestions of Dr. Donald B. McMullen, Walter Reed Army Institute of Research, are gratefully acknowledged.

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TABLE I. *Infection with* Dirofilaria uniformis *in young adult and suckling New Zealand white rabbits.*

Rabbit No.	Age of rabbit	No. of larvae injected	Microfilaremia days after injection	Maximum mf per 0.02 ml	Days after inoculation	Worms recovered at necropsy*			
						Male worms		Female worms	
						Number	Mean length mm	Number	Mean length mm
55	8 days	310	132	2	201	E	—	E	—
61	9 days	270	120	16	322	NF	—	NF	—
65	9 days	330	—	NF	308	NF	—	NF	—
72	13 days	885	116	33	319	14	13.3	35	27.5
6	14 days	50	—	NF	105	2	NR	11	22.8
4	14 days	150	134	6	232	4	12.5	10	31.4
1	15 days	300	112	12	153	11	11.3	29	23.0
5	25 days	600	123	< 1	196	80	12.7	89	27.1
2	31 days	400	117	2	196	E	12.1	E	23.2
228	10–12 weeks	245	—	NF	182	9	NR	19	NR
671	10–12 weeks	395	—	NF	140	9	NR	28	NR
672	10–12 weeks	420	—	NF	175	E	—	E	—
738	10–12 weeks	500	150	2	186	8	—	6	—
737	10–12 weeks	605	—	NF	169	E	—	E	—
1066	10–12 weeks	680	—	NF	78	E	—	E	—

* These figures do not include some worms which could not be counted because of partial disintegration. NF, not found; E, all worms encapsulated and disintegrated; NR, not recorded.

Since microfilaremias adequate to maintain the infection routinely in the laboratory were seldom attained in New Zealand white rabbits, another breed was tested for its susceptibility to this infection. Five suckling Dutch breed domestic rabbits were inoculated with varying numbers of infective *D. uniformis* larvae (table II).

TABLE II. D. uniformis *infection of 2- to 3- week-old Dutch rabbits.*

Rabbit No.	No. of larvae	Microfilaremia days after injection	Mf per 0.02 ml blood (Weeks after infection)							Worms recovered at necropsy				
			20	25	30	32	37	47	52	Days after inoculation	Male worms		Female worms	
											Number	Mean length	Number	Mean length
14	200	110	11	38	86	67	24	—	—	258	10	12.6	21	27.9
15	200	140	32	14	42	70	20	—	—	280	13	13.0	13	30.8
16	325	140	3	2	11	15	4	—	—	264	45	13.3	44	27.2
17	325	110	79	61	38	0	—	—	—	251	20	13.0	38	27.3
18	450	140	4	34	9	80	128	115	0	387	41	13.0	34	28.0

Within 140 days, each of these rabbits had a microfilaremia, and all but one developed a microfilarial count of more than 50 microfilariae per 0.02 ml of blood. One of these rabbits (No. 18) was used as a source of microfilariae for the infection of many groups of mosquitoes. In a second experiment, eight rabbits from two litters were given infective larvae. Five of these rabbits developed a microfilaremia within 5 months from the time of inoculation. Adult Dutch rabbits may also be susceptible to this infection since microfilariae appeared in the blood of the mother of one litter 133 days after she was exposed to the bite of infected mosquitoes.

On the basis of these results, it appears that Dutch rabbits are susceptible to infection with this parasite and may serve as suitable hosts for the routine maintenance of *D. uniformis*' life cycle in the laboratory.

SUMMARY

Attempts to infect guinea pigs, hamsters, rats, mice, and suckling mice with *Dirofilaria uniformis* were unsuccessful. The parasite developed in New Zealand white and mixed breed domestic rabbits, but relatively poor yields of microfilariae in the peripheral blood were obtained, and only for a comparatively short time. On the other hand, Dutch breed rabbits had sufficiently high microfilarial counts for periods long enough to ensure maintenance of the infection in the laboratory.

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DIAGNOSIS OF FILARIAL INFECTIONS IN DOGS. I. MICROFILARIAL SURVEYS*

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At least 11 species of filarial parasites have been reported from dogs in various regions of the world (Neveu-Lemaire, 1936; Chakravarty, 1936; Singh et al, 1958; Buckley, 1960). Only two of these have been demonstrated in dogs in the United States. The widespread occurrence of *Dirofilaria immitis* in the canine population of this country has been known for many years. Newton and Wright (1956, 1957) demonstrated the presence of another filariid, tentatively identified as *Dipetalonema reconditum*, in dogs from the vicinity of Maryland. On the basis of microfilarial types, various authors (Otto and Bauman, 1959; Bailey and Hoerlein, 1959; Rothstein et al, 1960) have given evidence that one or more additional species may be present in dogs in this country.

Most of the studies concerning prevalence of canine filarial infections in the United States were conducted before the presence of a species other than *D. immitis* was known. The apparently valid surveys were reviewed by Otto and Bauman (1959). More recent surveys have been reported by Rothstein et al (1960) and Wallenstein and Tibola (1960).

The present paper gives the results obtained in surveys of microfilarial infections in dog populations of certain coastal and inland cities in southeastern United States.

MATERIALS AND METHODS

The dogs included in these studies were from populations at four locations: Mobile, Ala.; Jacksonville, Fla.; Columbus, Ga.; and Auburn, Ala. Fifty dogs from 2 local pounds (Mobile and Prichard, Ala.) and a like number in two veterinary clinics comprised the dogs of the survey in the Mobile area. Similarly, 50 dogs from 2 pounds (Jacksonville City Pound and Duval County Pound) and 50 dogs in two veterinary clinics were examined at Jacksonville. The Columbus survey included 110 dogs from the pound in that city. The 100 dogs examined at Auburn were patients in the Small Animal Clinic at Auburn University and were owned by persons having local addresses (Lee County, Ala.).

The time of the survey at Mobile was June 28 to July 2, 1958, and that of the study at Jacksonville was December 8 to 12, 1958. All specimens from dogs in Columbus were examined during June, July and August 1958 and 1959, except 10 samples taken in September 1958. The study at Auburn was performed in the summer and fall of 1958 and 1959.

Dogs 1 year of age and older were randomly selected. The ages were obtained from case records or estimated by examination of the teeth (Merck and Co., Inc., 1955). The age, breed, sex, and hair length were recorded for each dog. The hair length was classified as either short or long. Dogs with hair comparable to that of Beagles or Pointers were considered as short-haired, whereas those with noticeably longer hair were designated as long-haired.

Approximately 5 ml of blood was collected from the cephalic vein of each animal and placed in a labeled and stoppered glass tube. Specimens from dogs at Mobile were allowed to clot, but those from dogs at other locations were drawn into syringes lined with a solution of heparin sodium (1,000 U.S.P. units per ml).

The blood samples were collected at irregular times of the day and examined during the same day. All examinations were performed according to the modified Knott method described by Newton and Wright (1956), with exception of the specimens at Mobile. Since those specimens were allowed to clot, cell-rich serum was examined. Also, 5.4 percent formalin was erroneously used in concentrating the microfilariae. In studies at all other locations whole blood containing heparin was mixed with 2 percent formalin. After the mixture prepared from each sample had been centrifuged (300 to 500 g) for 5 to 8 minutes, the tube was inverted to discard the supernatant fluid. The mouth of the inverted tube was touched to a clean paper towel to remove most of the remaining fluid. A drop of the sediment was transferred to a clean slide and mixed with a drop of 1:1,000 aqueous methylene blue; a coverslip was placed on the mixture.

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Precautions were taken to prevent contamination of each specimen with microfilariae from other samples. All glassware, syringes, and needles were washed and autoclaved (121 C for 15 to 20 minutes) prior to each use. Slides and coverslips were discarded after being used only once.

Each wet mount was examined with a compound microscope (100×). A sample was considered negative after failure to find a microfilaria in two mounts, which usually included more than half the sediment from 1 ml of blood or cell-rich serum. If microfilariae were found, the widths of as many as 10 and lengths of several were measured by using the high-dry objective (430×) and an ocular micrometer of the movable-hair type. The width was measured at approximately 50 to 60 microns from the anterior extremity of each microfilaria.

The presence or absence of the button-hooked tail described for *D. reconditum* (Newton and Wright, 1956) aided in making the tentative identifications. Other differential features were recognized early in the study and will be presented elsewhere.

RESULTS

Diagnosis of Microfilarial Infections

Only two distinct microfilarial types were discerned in blood samples from dogs of the four locations. The larger of these was tentatively identified as *D. immitis* and the smaller as *D. reconditum*.

The data on width measurements of microfilariae from the different groups are summarized in table I. The number of microfilariae measured, the range of measurements, and the mean width along with its standard error are given for microfilariae from each group of dogs. Measurements for the two microfilarial types were compared by Student's *t* test; highly significant differences were obtained.

Statistical comparisons were made to determine whether differences existed among the width measurements of *D. immitis* microfilariae from the different sources. The measurements of microfilariae from dogs of the Mobile pounds were significantly different from those of the Mobile clinics, as shown by the *t* test. Similarly, width measurements for microfilariae of *D. immitis* from the two sources in Jacksonville were found to be different. Width measurements for microfilariae of *D. immitis* were not significantly different if the data were treated as four groups and analyzed by the *F* test. When the data of all six possible sources were treated separately, the *F* test revealed highly significant difference. However, if data from the Mobile clinics were excluded and width measurements for the five other sources compared by the *F* test, there was no statistical difference.

Statistical analysis showed that width measurements for microfilariae of *D. reconditum* from all animal groups were similar except for the two groups at Mobile. If either of these were included in the analysis, a significant difference was obtained by the *F* test. With this test no difference was found when the two Mobile groups were excluded. Furthermore, a highly significant difference was obtained by using the *t* test to compare width measurements of *D. reconditum* from dogs of the Mobile clinics with those from dogs of the Mobile pounds.

The data on length measurements for microfilariae of *D. immitis* and *D. reconditum* are given in table II. Measurements for the two kinds of microfilariae from each group of animals were compared by use of the *t* test; highly significant difference was found between the two types.

Statistical analyses gave evidence of limited homogeneity among the length measurements for microfilariae of *D. immitis* from different groups of dogs. No significant difference was found when the two groups at Jacksonville were compared by the *t* test. By similar analysis there

TABLE I. *Width measurements of microfilariae from dogs at four locations.*

Location	*Dirofilaria immitis*				*Dipetalonema reconditum*		
	n	Range (microns)	Mean ± SE (microns)	"t"	n	Range (microns)	Mean ± SE (microns)
Mobile, Ala.							
Pounds	239	6.1–7.2	6.74 ± 0.016	30.7*	37	4.9–5.8	5.38 ± 0.045
Clinics	158	6.5–7.2	6.84 ± 0.012	44.4*	42	4.7–5.8	5.52 ± 0.033
Jacksonville, Fla.							
Pounds	48	6.5–6.9	6.76 ± 0.017	64.1*	83	4.9–5.6	5.23 ± 0.016
Clinics	137	6.3–6.9	6.72 ± 0.007	31.9*	46	5.2–5.4	5.20 ± 0.014
Columbus, Ga.							
Pound	30	6.5–6.9	6.75 ± 0.022	59.6*	265	4.9–5.4	5.20 ± 0.008
Auburn, Ala.							
Clinic	17	6.5–6.7	6.69 ± 0.019	34.1*	141	4.9–5.8	5.19 ± 0.014
All Groups	629	6.1–7.2	6.76 ± 0.007	89.3*	614	4.7–5.8	5.23 ± 0.015

* Highly significant ($P < 0.001$).

TABLE II. *Length measurements of microfilariae from dogs at four locations.*

Location	*Dirofilaria immitis* n	Range (microns)	Mean ± SE (microns)	"t"	*Dipetalonema reconditum* n	Range (microns)	Mean ± SE (microns)
Mobile, Ala.							
Pounds	70	285.6–336.0	310.2 ± 1.23	—	1	—	246.4
Clinics	42	292.9–336.0	314.5 ± 1.47	12.8*	9	257.6–291.2	268.8 ± 3.54
Jacksonville, Fla							
Pounds	17	299.5–329.1	314.8 ± 1.74	19.7*	19	257.6–280.0	269.7 ± 1.55
Clinics	56	298.1–336.0	314.1 ± 1.29	14.0*	10	260.5–286.5	268.8 ± 2.53
Columbus, Ga.							
Pound	13	311.1–339.8	325.7 ± 2.50	16.0*	25	262.8–291.6	274.2 ± 1.90
Auburn, Ala.							
Clinic	5	315.8–327.7	320.9 ± 0.21	12.4*	13	257.6–287.6	271.3 ± 2.04
All Groups	203	285.6–339.8	313.8 ± 0.72	32.3*	77	246.4–291.6	270.9 ± 1.04

* Highly significant ($P < 0.001$).

was no significant difference between measurements for microfilariae of *D. immitis* at Auburn and Columbus. But, several other analyses were made using both *t* and *F* tests, and all resulted in significant differences.

On the basis of statistical analysis, all groups of length measurements for microfilariae of *D. reconditum* were similar with exception of the single observation recorded for the Mobile pounds. Data from the five other groups were compared by the *F* test which revealed no difference.

The tail configuration was observed on each microfilaria of *D. reconditum* for which the width measurement was recorded. The button-hooked tail described for this species by Newton and Wright (1956) was recorded for 202 (33 percent) of the 614 microfilariae. No doubt this character existed on a greater percentage of the microfilariae, but the full length of the tail was frequently not visible because of positioning or the presence of cellular debris.

Prevalence of Microfilarial Infections

The prevalence of microfilarial infections in dogs surveyed at the four locations is given in table III. The average age of animals in each group is also given.

More than half of the animals from Mobile, Jacksonville, and Columbus were found to be harboring microfilariae, whereas only 34 percent of those at Auburn were infected. The greatest incidence was found at Mobile where 58 percent of the animals had microfiliariae. The predominant filariid in dogs of Mobile was *D. immitis*, but at other locations *D. reconditum* was more prevalent.

Although noticeable differences were observed in the incidence of *D. reconditum*, this filariid was quite prevalent in dogs of all locations surveyed. At Mobile, 23 percent of the animals harbored this parasite; it was found in 37, 52, and 32 percent of the dogs at Jacksonville, Columbus, and Auburn, respectively.

Infections of *D. immitis* were diagnosed for 24 percent of all pound animals and 18 percent of those from veterinary clinics. The incidence of *D. reconditum* was 44 percent of the 210 pound animals and only 28 percent of the 200 animals from clinics. Fifty-seven percent of all pound animals were infected and 42 percent of

TABLE III. *Prevalence of microfilarial infections in dogs at four locations.*

Source and (number) of dogs	Average age, years	*D. immitis* only	*D. reconditum* only	Mixed infections*	Total dogs infected
		(pct.)	(pct.)	(pct.)	(pct.)
		Mobile, Alabama			
Vet. clinics (50)	4.2	14 (28)	9 (18)	2 (4)	25 (50)
Dog pounds (50)	4.5	21 (42)	7 (14)	5 (10)	33 (66)
Total (100)	4.3	35 (35)	16 (16)	7 (7)	58 (58)
		Jacksonville, Florida			
Vet. clinics (50)	4.7	13 (26)	9 (18)	3 (6)	25 (50)
Dog pounds (50)	4.1	4 (8)	22 (44)	3 (6)	29 (58)
Total (100)	4.4	17 (17)	31 (31)	6 (6)	54 (54)
		Columbus, Georiga			
Dog pound (110)	4.9	2 (2)	55 (50)	2 (2)	59 (54)
		Auburn, Alabama			
Vet. clinic (100)	4.5	2 (2)	31 (31)	1 (1)	34 (34)

* *D immitis* and *D. reconditum*.

all dogs from clinic sources harbored microfilariae of one or both species.

The incidence of microfilarial infections were quite similar for dogs in veterinary clinics at Mobile and Jacksonville. Infections of *D. immitis* were diagnosed for 32 percent of dogs from each of the two sources. Infections of *D. reconditum* were found in 22 percent of dogs from veterinary clinics in Mobile and in 24 percent of a similar group at Jacksonville. Fifty percent of animals in each group were found to be infected with microfilariae.

No report was found to mention a difference in prevalence of filarial infections among dogs from separate sources in a restricted locality such as a city or county. The following data suggest such a difference for animals of two sources in Jacksonville. Microfilariae of *D. immitis* were identified in blood from 16 (32 percent) of 50 dogs from veterinary clinics and only 7 (14 percent) of 50 dogs from the local pounds. When these data were tested by the chi-square method, a value representing significant deviation from the expected distribution was obtained. Therefore, the incidence of heartworm infection in the two groups was statistically different.

The incidences of *D. reconditum* also differed at Jacksonville. Microfilariae of this species occurred in 24 percent of the dogs from clinic sources as compared to 50 percent of those from pounds. Analysis by the chi-square method gave a highly significant value.

The dogs included in these studies were separated into five age classes, namely, 1 to 3, 3 to 5, 5 to 7, 7 to 9, and 9 or more years old. The number of animals harboring each microfilarial species and the total number of infections were determined for the individual classes. These data are given in figure 1. The infections are expressed as percentages of animals composing each age class. The total number of dogs in each class is also given.

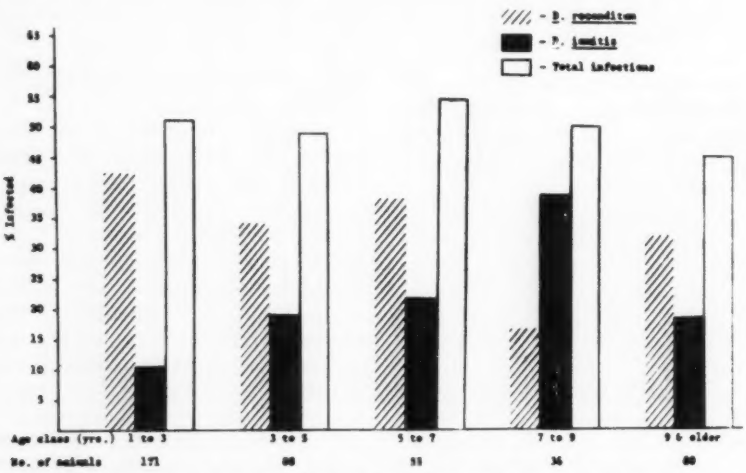

FIGURE 1. Distribution of microfilarial infections among age classes of all dogs.

The incidence of *D. immitis* infection gradually increased with age up to about the 7th to 9th year and decreased in older animals. In general the opposite trends occurred in distribution of *D. reconditum*. Total microfilarial infections were rather constant in the different age classes but decreased slightly among older animals.

The distribution of infections among age classes of clinic dogs was compared with that of pound dogs. The general relationships presented in figure 1 were observed, but in clinic animals the greatest incidence of *D. immitis* occurred in dogs 5 to 7 years old. Also, the incidence of *D. reconditum* decreased with increasing age and reached its lowest point in clinic animals of 9 years and older.

A total of 72 infections of *D. immitis* was found among the 410 dogs. Forty-six (20 percent) of 235 male dogs were infected with this

filariid as compared to 26 (15 percent) of 175 females. Analysis of these data by the chi-square method showed no difference between the two sexes.

One hundred and nine (46 percent) of the 235 males were infected with microfilariae of *D. reconditum*, but only 40 (23 percent) of 175 females harbored this species. When these data were tested by the chi-square method, it was found that *D. reconditum* was significantly more prevalent among male dogs than females. The average age of infected males was 4.1 years compared to 4.3 years for the infected females.

The hair length was determined for 304 of the dogs included in the study. Twenty (12 percent) of the 172 dogs with short hair and 10 (8 percent) of the 132 long-haired dogs had microfilariae of *D. immitis*. There was no difference between the groups on the basis of chi-square analysis.

Infections of *D. reconditum* were demonstrated in 68 (40 percent) of 172 short-haired dogs and 57 (43 percent) of 132 long-haired dogs. Analysis by the chi-square method showed no difference in prevalence of the infection.

The findings on prevalence of microfilarial infections in dogs of the various breeds are given in table IV. The animals are divided into five major groups partly on the basis of classifications used by the American Kennel Club. The average age of each group is given.

The prevalence of *D. immitis* ranged from 18 to 23 percent among mongrels, working dogs, and sporting dogs, but was much less in other groups. Infections of *D. reconditum* were prevalent in all groups. Total microfilarial infections occurred with decreasing frequency among the groups listed from 1 through 5. Fifty-six percent of mongrels were infected with microfilariae as compared with only 27 percent of the toy breeds.

TABLE IV. *Prevalence of microfilarial infections in dogs of various breeds.*

Breed	*D. immitis*	*D. reconditum*	Infected	Total dogs
Group 1. Mongrels* Av. age 4.6 yrs				
Totals (pct.)	33 (18)	77 (43)	101 (56)	180
Group 2. Working dogs Av. age 4.3 yrs				
Boxers	4	4	8	21
Collies*	4	11	14	25
Dobermans	1	0	1	1
Shepherds*	4	8	11	20
Gr. Pyrenees	0	1	1	1
Totals (pct.)	13 (19)	24 (35)	35 (51)	68
Group 3. Sporting dogs Av. age 4.9 yrs				
Pointers	10	6	16	19
Retrievers	0	0	0	2
Setters	0	2	2	5
Spaniels*	9	13	21	40
Weimaraners	0	1	1	6
Hounds*	4	7	9	20
Dachshunds	0	1	1	8
Totals (pct.)	23 (23)	30 (30)	50 (50)	100
Group 4. Non-sporting and terrier breeds Av. age 4.6 yrs				
Bostons	0	1	1	5
Bulldogs	0	1	1	3
Chows*	1	3	3	6
Dalmatians	0	1	1	3
Poodles	0	0	0	4
Terriers*	2	9	10	30
Totals (pct.)	3 (6)	15 (30)	16 (31)	51
Group 5. Toy breeds Av. age 2.9 yrs				
Chihuahuas	0	2	2	5
Pekingese	0	1	1	4
Pomeranians	0	0	0	1
Pugs	0	0	0	1
Totals (pct.)	0	3 (27)	3 (27)	11
GRAND TOTALS	72	149	205	410

* One or more animals infected with both types of microfilariae

DISCUSSION

The validity of the two microfilarial types observed in blood specimens from dogs at the four locations was supported by statistical analyses. Measurements of the two species always differed at a high level of significance when compared by the *t* test.

In some instances significant differences were found among the width and length measurements of each microfilarial type. These differences were usually due to data from one or both of the animal groups at Mobile. Since that population was the first surveyed, the data may have been influenced by lack of experience in making the measurements. Furthermore, the mounts were not prepared in the identical manner employed at other locations. No explanation is offered as to why so little homogeneity was found among the length measurements for microfilariae of *D. immitis* from the different groups.

There was some uncertainty about identification of one microfilaria observed in blood from a pound dog at Mobile. It was 246.4 microns long, which was 11 microns less than any other microfilaria measured. It was 5.4 microns wide and the tail ended in the button-hooked configuration characteristic of *D. reconditum*. Seven other microfilariae from the same dog had widths well within the range recorded for *D. reconditum*. For these reasons, the microfilaria in question was identified as *D. reconditum*.

The microfilarial measurements obtained in the present study are compared with those of Newton and Wright (1956) in table V. The major discrepancy between the two sets of data involves width measurements for microfilariae of *D. reconditum*. Other measurements are in general agreement except for the greater ranges of measurements obtained in the present study.

TABLE V. *Comparison of microfilarial measurements* with those of a previous study.*

Source of data	*D. immitis*				*D. reconditum*			
	Width		Length		Width		Length	
	n	Av. (range)	n	Av. (range)	n	Av. (range)	n	Av. (range)
Newton and Wright, 1956	20	6.9 (6.7–7.1)	20	313 (307–322)	20	4.6 (4.3–4.8)	20	276 (269–283)
Present study	629	6.8 (6.1–7.2)	203	314 (286–340)	614	5.2 (4.7–5.8)	77	271 (246–292)

* All measurements in microns.

Newton and Wright (1957) were of the opinion that *D. reconditum* is the common filariid of dogs in northern United States and that both *D. reconditum* and *D. immitis* are prevalent in the southern part of the country. The data of the present study partially support their belief and suggest that *D. reconditum* may be the predominant filariid in dogs of the South also. This species was found more frequently in each of the populations surveyed except the one at Mobile. Moreover, the high incidence of *D. immitis* and the associated large numbers of microfilariae in the blood from dogs at Mobile may have reduced the efficiency of demonstrating microfilariae of *D. reconditum* which usually occurred in low concentrations.

Infections of *D. immitis* were found in 42 percent of the dogs at Mobile and 23 percent of those surveyed at Jacksonville. Except for the data on dogs of the Jacksonville pound where only 14 percent were infected, these findings generally agree with those of Brown (1939) at Savannah, Ga.; Hinman (1935) and Hinman and Baker (1936) at New Orleans, La.; and Ward and Franklin (1953) at Clarksdale, Miss.

Heartworm infections were encountered much less frequently at the inland cities of Auburn and Columbus where the incidences were 3 and 4 percent, respectively. These findings compare favorably with the percentages of dogs from which adult *D. immitis* were recovered by Eyles et al (1954) at Memphis, Tenn. and by Mundhenk and Greene (1939) at Columbus, Ga. and Montgomery, Ala.

The incidence of total microfilarial infections at Auburn was 34 percent, which was very similar to findings of a previously unreported survey of the canine population in this area. Data compiled by Folse (1950) indicate that 37 percent of 99 mature dogs in the Small Animal Clinic at Auburn University harbored microfilariae.

The significant difference in incidence of *D. immitis* in pound dogs and dogs of veterinary clinics in Jacksonville may represent an actual difference in occurrence of this infection in dogs of separate sources within a small locality. The average age of pound animals was 4.1 years, while that of clinic animals was 4.7 years; age, therefore, does not seem to be a significant factor.

A local veterinarian who supervises the care of dogs at one of the pounds stated that a large percentage of the pound animals was collected from an area of dense human population in downtown Jacksonville where little vegetation is present. By comparison, most of the dogs in veterinary clinics probably originated in the outlying districts of the city where there is less dense human population and an abundance of mosquito cover such as trees and shrubs. It seems that the latter areas would provide more suitable conditions for transmission of *D. immitis*. Information regarding the habits of those mosquito species which serve as vectors of the infection in Jacksonville would be needed to confirm or disprove this possibility.

The epidemiology of *D. immitis* in Jacksonville may be similar to that which Otto and Jachowski (1952) observed for *Wuchereria bancrofti* in American and Western Samoa. They found the "index of potential transmismission" to be generally greater in the bush, or in houses near the bush, because of the breeding and resting habits of the vector, *Aedes pseudoscutellaris*.

Prevalence of the flea intermediate hosts may have been responsible for the highly sig-

nificant difference in incidence of *D. reconditum* observed between clinic and pound dogs at Jacksonville.

Younger animals always had the lowest incidence of *D. immitis* and the greatest incidence of *D. reconditum*. This provides strong evidence against the possibility of prenatal infection suggested for *D. immitis* (Hinman, 1935; Augustine, 1938). On the other hand, it does not prove the occurrence of prenatal infection by *D. reconditum*. It may be simply that young dogs are more frequently exposed to fleas than to mosquitoes.

The incidence of *D. immitis* increases with age of dogs until about the 7th year, after which there is a decline in percentage of natural infections. Similar findings were reported by Wallenstein and Tibola (1960). The increasing incidence may be a simple process of accumulating infected animals. The wane of incidence during senescence is most likely the result of less exposure to the intermediate host, either imposed by the owners or by aging per se. Also, immune mechanisms of the host may be involved.

The data were highly convincing that *D. reconditum* occurs more frequently in males than in females. A comparative rate of approximately 2:1 was found even though the average ages of the two groups were practically the same. This almost certainly indicates difference in susceptibility of the two sexes of dogs. Data reported by Wallenstein and Tibola (1960) gave a ratio of 1.5:1 in favor of the infection in males. Ehrenford (1956) has reported a similar distribution of *Toxocara canis* in dogs of different sexes.

There was no significant difference between incidence of *D. immitis* in males and females. This is at variance with a recent report (Wallenstein and Tibola, 1960) in which males were found to be infected with heartworms four times as frequently as females.

Various authors (Hinman and Baker, 1936; Augustine, 1938; Brown, 1939) have indicated that *D. immitis* is unequally distributed in animals having different lengths of hair. In the present study, neither *D. immitis* nor *D. reconditum* was found to be more prevalent in short- or long-haired dogs.

The prevalence of both filariids varied considerably among the different breeds of dogs. This is probably related to care and use of the dogs rather than difference in susceptibility. The present study indicates that *D. reconditum* is more widely distributed than *D. immitis* among the different breeds.

SUMMARY

The modified Knott method was used to survey the microfilarial infections in mature dogs at each of four locations: Mobile, Ala., Jacksonville, Fla., Columbus, Ga., and Auburn, Ala. Only two distinct microfilarial types were observed. They were tentatively identified as *Dirofilaria immitis* and *Dipetalonema reconditum*. Measurements for the two types were compared by the *t* test and the difference was found to be highly significant.

The incidence of microfilarial infections was found to differ appreciably among the various populations, but both species were always present. Total microfilarial infections ranged from 34 to 58 percent among dogs of the four locations. Infections of *D. reconditum* predominated at each location except Mobile, suggesting that this species is quite prevalent in southeastern United States.

The prevalence of each mircofilarial species was found to vary among animals of different ages. Infections of *D. immitis* were observed with increasing frequency in animals up to the age of about 7 years; in older animals the incidence was noticeably reduced. Essentially the opposite trends were seen in prevalence of *D. reconditum* among the different age classes.

The prevalence of *D. immitis* did not vary significantly according to sex or hair length of the animals. The incidence of *D. reconditum* did not differ among dogs with different hair lengths. However, *D. reconditum* occurred in male dogs twice as frequently as in females; the difference was highly significant. The prevalence of both filariae varied considerably among dogs of the different breeds.

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ERRATA

June 1961 issue, p. 414, right column, paragraph 3. Interchange line 3 starting "hippoboscids" with line 4 starting "(*Myrsidea picae*)."

August 1961 supplement, p. 1, first title of program. Substitute "S. G. Zam, L. J. Thomas. Jr. and W. E. Martin" for "Zein-Eldin, E. Ahmad and Scott, J. Allen."

August 1961 supplement, p. 22, line 3. Read "unsuccessful" for "successful."

August 1961 supplement, p. 44, abstract No. 98, line 11. Read "pubic" for "public."

DIPETALONEMA WITEI, FILARIAL PARASITE OF THE JIRD, *MERIONES LIBYCUS*. II. THE REPRODUCTIVE SYSTEM, GAMETOGENESIS AND DEVELOPMENT OF THE MICROFILARIA

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The filarial parasite *Dipetalonema witei* is found naturally infecting the Libyan jird *Meriones libycus;* the adult worms live in the subcutaneous tissues and external muscle layers; the microfilariae circulate in the blood and are transmitted by the argasid tick *Ornithodorus tartakovskyi.*

The parasite is easily maintained in the laboratory, (Baltazard et al (1953); Worms, Terry and Terry (1961)) and provides suitable material for morphological studies. This paper describes the reproductive system, gametogenesis, and development of the microfilariae.

METHODS

Adult worms were removed from freshly-killed jirds, fixed with corrosive acetic fixative, and sectioned either transversely or longitudinally at 10 microns. Alternate slides were stained with hematoxylin and eosin or Giemsa.

OBSERVATIONS

Male Reproductive System

Component parts. The male reproductive sytem of *D. witei* consists of a single, anterior, uncoiled testis and its associated duct which passes posteriorly without convolutions and opens into the ventral side of the cloaca. Except for the extreme posterior part, both the testis and the duct are associated with one of the prominent lateral lines.

The tip of the testis is close to the junction of the intestine and the pharynx. The associated duct is differentiated into a short vas efferens, a long dilated vesicula seminalis and a short vas deferens and ejaculatory duct contained within the coils of the tail. The ejaculatory duct opens into the cloaca a short distance anterior to the ventral opening. Originating dorsal to, and projecting into the cloaca, is a pair of unequal spicules, the right being longer and grooved with annular thickenings, the left being small and simple (plate I, fig. 1).

Wall of the reproductive system. The tip of the testis is curved and is occupied by a large, clear "cap cell." This cap cell is continuous with the flattened epithelium covering the testis (plate I, fig. 2) and the epithelium of the duct. In the vas efferens the epithelium is cuboidal and the cells contain massive inclusions which are presumably the absorbed residual cytoplasm left after the formation of the spermatozoa (plate I, fig. 3). The epithelium covering the vesicula seminalis is much more flattened (plate I, fig. 4). The epithelium of the vas deferens is again cuboidal (plate I, fig. 5); the cells have a glandular appearance and are probably concerned with secreting the fluid in which the spermatozoa are suspended. The cloacal epithelium consists of a few large cells incompletely lined with a thin cuticle.

The wall of the vas deferens contains a thin, poorly defined layer of muscle fibers. This layer, however, is much more definite in the wall of the ejaculatory duct (plate I, fig. 6).

The spicules which run across the cloaca are enclosed in part in a spicular sac. Associated with the spicules are muscle cells which extend posterior to the cloacal opening.

Development of the spermatozoa. The anterior part of the testis is occupied by a syncitial mass containing scattered nuclei. Passing posteriorly the number of nuclei increases, showing that this region is a multiplication zone (plate II, fig. 1). As in the female, few metaphase plates are seen so that the nuclear division must be rapid. At the end of this zone distinct masses of cytoplasm aggregate round each nucleus and the volume of both nuclei and cyto-

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plasm increases to give cubical spermatogonia (plate II, fig. 2). The spermatogonia are arranged more or less radially but there is no distinct central rachis. During the multiplication phase the nuclei contain scattered chromatin; later they show a clear nuclear sap with a single prominent nucleolus.

In the following maturation phase chromatin material appears at the periphery. The nuclear membrane disappears and six composite chromosome units become visible (plate II, fig. 3). Two successive divisions follow yielding spermatids containing either five or six chromosomes (plate II, figs. 4 and 5). Cytoplasmic reduction then takes place (plate II, fig. 6). Up to this point the whole cytoplasm is basophilic but following this reduction, the cytoplasm of the formed spermatozoa becomes acidophilic. The residual cytoplasm remains basophilic and appears to be absorbed by the epithelium of the vas efferens.

The spermatozoa are at first spherical (3 by 2.5 by 2.5 microns) and contain five or six basophilic chromosomes. As they enter the vesicula seminalis they become more elongate (1.5 by 1.5 by 8 microns) and often there is a "lining-out" of the chromosomes (plate II, fig. 7).

The spermatozoa are stored in the vesicula seminalis; later they pass into the vas deferens where they are suspended in a basophilic fluid. At this stage they are still elongate but when they are present in the fertilization chamber of the female they have become spherical again.

Female Reproductive System

Component parts. The female reproductive system of *D. witei* is didelphic and opistodelphic. There are paired ovaries, oviducts and uteri, and a single vagina and vulva. The more posterior ovary terminates at the level of the anus, the other ovary ends about 3 mm anterior to the anus (plate III, fig. 1). The ovaries are coiled and occupy only a small part of the total length of the worm. Each is succeeded by a short S-shaped oviduct, about 2.5 mm in length, which widens abruptly into the uterus. The posterior part of the uterus acts as a fertilization chamber (plate III, fig. 2). The paired uteri (plate III, fig. 3) occupy by far the greater part of the length of the worm and pass without convolutions to the level of the vulva near the glandular region of the oesophagus. In this region the uteri become convoluted, describing loops anterior and posterior to the vulva. The two uteri become bound in a common sheath for a short distance before they join the single vagina. The vagina passes forward to the level of the anterior region of the muscular oesophagus and then turns posteriorly before entering the muscular mass of the vulva. The vagina describes a spiral path through the vulva and opens to the exterior in a shallow depression in the mid-ventral line (plate III, fig. 4).

The wall of the reproductive system. The tip of the ovary is occupied by a large eccentrically placed cap cell which is continuous with the flattened epithelium covering the ovary. This epithelium has a conspicuous basement membrane but does not appear to have associated muscle fibers. The epithelium varies from the squamous type adjacent to the cap cell to a more cuboid form in the anterior region.

The wall of the oviduct is formed from columnar epithelial cells. The basement membrane is prominent and in a few sections there are indications of the presence of a thin and ill-defined muscle layer. The general appearance of the epithelium is that often associated with a glandular function and it may be that the cells play a part in secreting the egg membrane. On the other hand, the cells adjacent to the uterus appear to have ingested spermatozoa.

The uterus is lined with a flattened epithelium which is continuous with the epithelium of the oviduct. The cells of the uterus epithelium may, however, bulge into the lumen when the uterus is relaxed. The basal membrane is prominent and muscle fibers are present. Where the uteri become bound in a common sheath, the epithelial cells are high and irregular with vacuoles on their free border; there is an obvious outer muscular sheath. The vaginal epithelium is similar in appearance and the muscles show transverse striations. The vulva consists of a mass of muscle which is continuous posteriorly with that of the body wall. The epithelium lining the convoluted passage through the vulva is similar to that of the vagina. The cuticle of the body wall appears to extend for only a short distance into the vulva.

Development of the oocytes. The tip of the ovary is occupied by syncytial mass independent of the cap cell. The nuclei are at first scattered but soon become closely packed and show distinct nuclear membranes and well-marked chromatin masses (plate IV, fig. 1). Passing an-

teriorly the number of nuclei increases as the ovary widens, showing that this part of the ovary is a multiplication region. Nuclear divisions must, however, be rapid as few metaphase plates can be observed. At the end of the multiplication phase the nuclei become surrounded by discrete masses of cytoplasm, giving cubical oogonia (plate IV, fig. 2). These are arranged in strings in an irregular radial fashion; there is no sign of a central rachis. Each oogonium has a spherical nucleus with a single prominent nucleolus.

The succeeding growth region is much longer than the multiplication region. Here the strings of cubical oogonia are transformed into clusters of elongate primary oocytes (plate IV, fig. 3); the nucleus of the oocyte also becomes elongate. At the anterior end of the ovary, adjacent to the oviduct, the oocytes lie freely within the oviduct.

The oocytes pass singly through the narrow U-shaped oviduct into the fertilization chamber. During their passage through the oviduct an outer secretion or delicate membrane is added.

Oogenesis and fertilization. The fertilization chamber in the distal part of the uterus contains a mass of small spermatozoa. These are acidophilic with a number of basophilic inclusions but no obvious nucleus. Fertilization occurs with the penetration of the whole spermatozoon at one pole of the nucleus. The spermatozoon then becomes indistinct, the nuclear membrane of the oocyte disappears and the chromosomes appear at the pole opposite to the point of entry of the sperm (plate IV, fig. 4). The chromosomes separate and the first polar body is formed (plate IV, figs. 5 and 6). The terminal group of chromosomes divides again and the second polar body is formed. On occasions the first polar body also divides again. At this stage the vitelline membranes are apparent but the ovum shows no obvious detailed structure except for one or two indistinct masses which are presumably the male and female pronuclei (plate IV, figure 7).

Development of the microfilariae. Cleavage begins immediately after fertilization and the metaphase plates are numerous (plate IV, figs. 8 and 9). The chromosomes are not very distinct and it was impossible to count them accurately; however, there was no obvious diminution of chromatin material. Early cleavage appears to be rapid (plate V, fig. 1), but the later developmental stages occupy a considerable part of the uterus. Moving anteriorly, invagination occurs producing the typical gastrulae (plate V, fig. 2). These gastrulae then elongate to give the sausage-shaped embryos which coil within their membranes (plate V, fig. 3). Later, the young microfilariae straighten and are freed from the membranes (plate V, fig. 4). They move forward to the proximal part of the uterus where they are tightly packed with their long axes orientated along the length of uterus.

At all levels, one uterus contains more mature forms than the other and throughout both there are embryos which fail to differentiate, and remain as rounded masses of cells, often with pycnotic nuclei.

DISCUSSION

The female reproductive system of *D. witei* conforms to the general pattern described for other members of the sub-order *Spirurina* (Chitwood and Chitwood, 1940), and is essentially similar to that of the other two members of the super-family *Filarioidea* which have been so far described, *Litomosoides carinii* (Cross and Scott, 1947) and *Dirofilaria corynodes* (= *aethiops*) (Webber, 1955). *D. witei* does, however, appear to differ from *Dirofilaria corynodes* in that the ovary has no central rachis. The cap cell in *D. witei* is clearly part of the epithelium covering the ovary and is quite distinct from the anterior ovarian tissue.

As is general in parasitic nematodes the female reproductive system is complex, but in this species coiling is confined to the distal part of the ovaries and the proximal part of the uteri.

The male reproductive system is of a well-differentiated monorchic form with clearly vas efferens, vesicula seminalis, vas deferens, and ejaculatory duct. The system differs from that described for other monorchic forms in the virtual absence of flexure of the testis. Only the extreme tip of the testis in the region of the cap cell shows slight bending. *Dirofilaria corynodes* is known to be similar in this respect (authors' unpublished observations). Chitwood and Chitwood (1940) have described the phasmids as typically having a chord of six or more radiating series of cells attached to a central rachis within the growth region of the testis. This structure was not observed in *D. witei;* here the cells were scattered and there was no

rachis.

Gametogenesis in the female of *D. witei* is essentially similar to that described for *Rhabdias bufonis* (Chitwood and Chitwood (1940)).

Penetration of the sperm at one pole of the oocyte is followed by reduction division and the extrusion of the polar bodies at the opposite poles. Spermatogenesis in *D. witei* differs however from previously described forms in that no metamorphosis of the spermatid occurs other than a reduction in cytoplasm. There is no indication of an acrosome or any of the other structures reported to occur in nematode spermatozoa.

The male is the heterozygotic sex and produces spermatozoa of two types. These differ only in their number of chromatin units, 5 or 6. These units persist as separate entities instead of becoming enclosed in a nuclear membrane. This persistence of visible chromosomes has also been observed in *Dirofilaria corynodes* (authors' unpublished observations) and *Litomosoides carinii* (Taylor, 1960), and has also been reported to occur in *Ancyranthus cistidicola* (Mulsow, 1912) which is also a member of the suborder Spirurina. This phenomenon may possibly be widespread within the group. In *Ancyranthus cystidicola* there is an XO mechanism of sex determination. The sperm are of two types (n 5 or 6); the X chromosome is unpaired and wanders to one pole or the other during the first meiotic division. A similar mechanism appears to exist for *D. witei* but this has not been conclusively demonstrated.

The development of the microfilaria from the fertilized egg appears to be identical with that reported for *Dirofilaria corynodes* (Webber, 1955) and *Litomosoides carinii* (McFadzean and Smiles, 1956; Taylor, 1960).

SUMMARY

The anatomy of the male and female reproductive systems of *Dipetalonema witei* is described and an account given of gametogenesis and development of the microfilariae.

Reproduction in this parasite conforms to the general pattern described for other members of the Spirurina.

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EXPLANATION OF PLATES

Dipetalonema witei

Abbreviations used: e.d., ejaculatory duct; f.c., fertilization chamber; int., intestine; l. sp., left spicule; oes, oesophagus; ov., ovary; re., rectum; r.sp., right spicule; te., testis; ut., uterus; va., vagina; v.d. vas deferens; v.e., vas efferens; v.s., vesicula seminalis; vu., vulva.

PLATE I

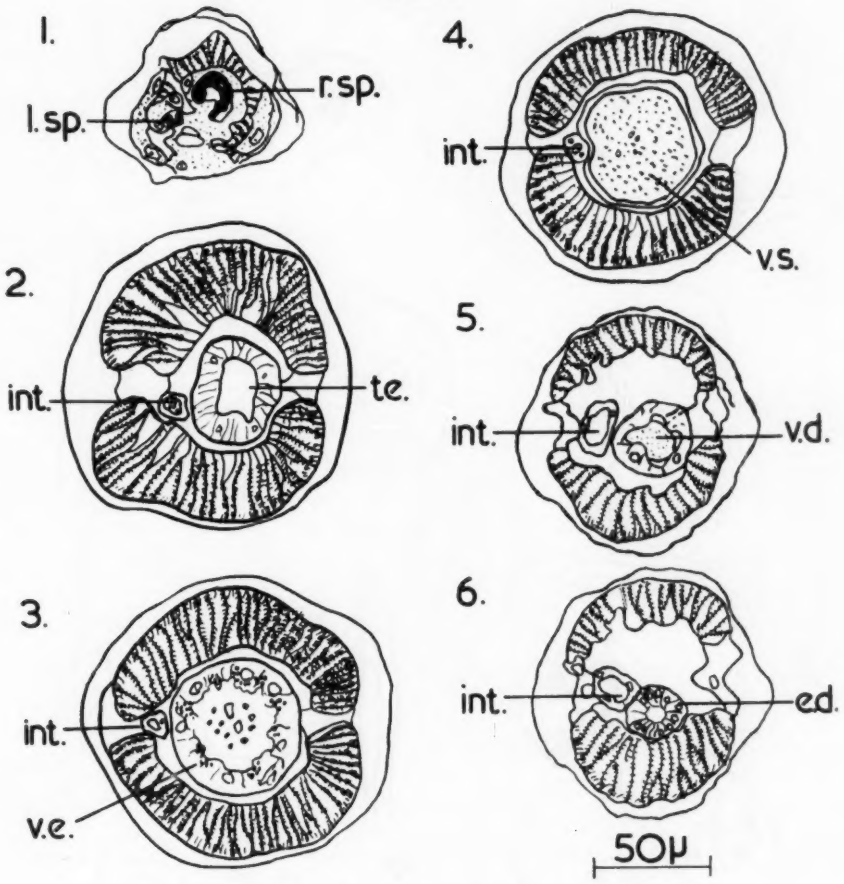

PLATE I. The male reproductive system.

FIGURE 1. Transverse section of cloacal region showing spicules.
FIGURE 2. Transverse section of anterior region of testis.
FIGURE 3. Transverse section of region of vas efferens showing absorption of residual cytoplasm.
FGIURE 4. Transverse section in region of vesicula seminalis.
FIGURE 5. Transverse section in region of vas deferens.
FIGURE 6. Transverse section in region of ejaculatory duct.

PLATE II

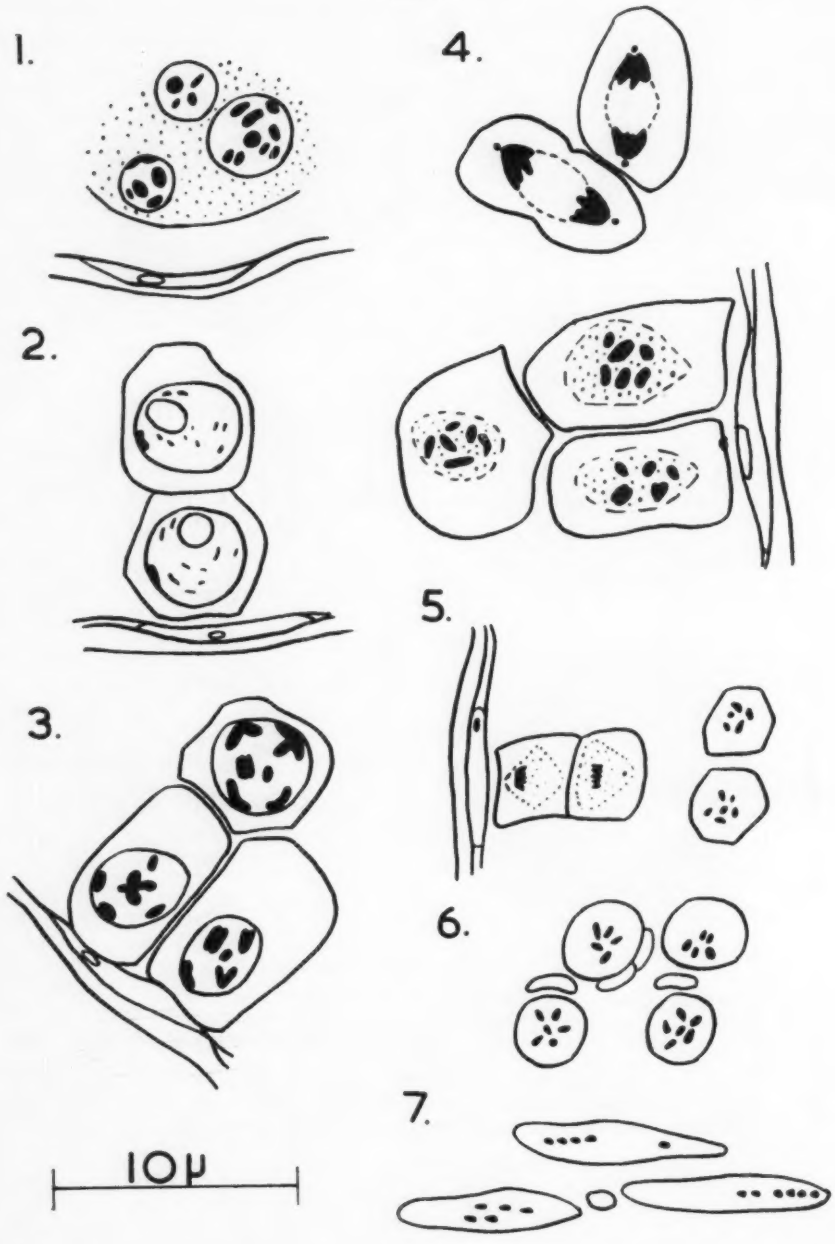

PLATE III

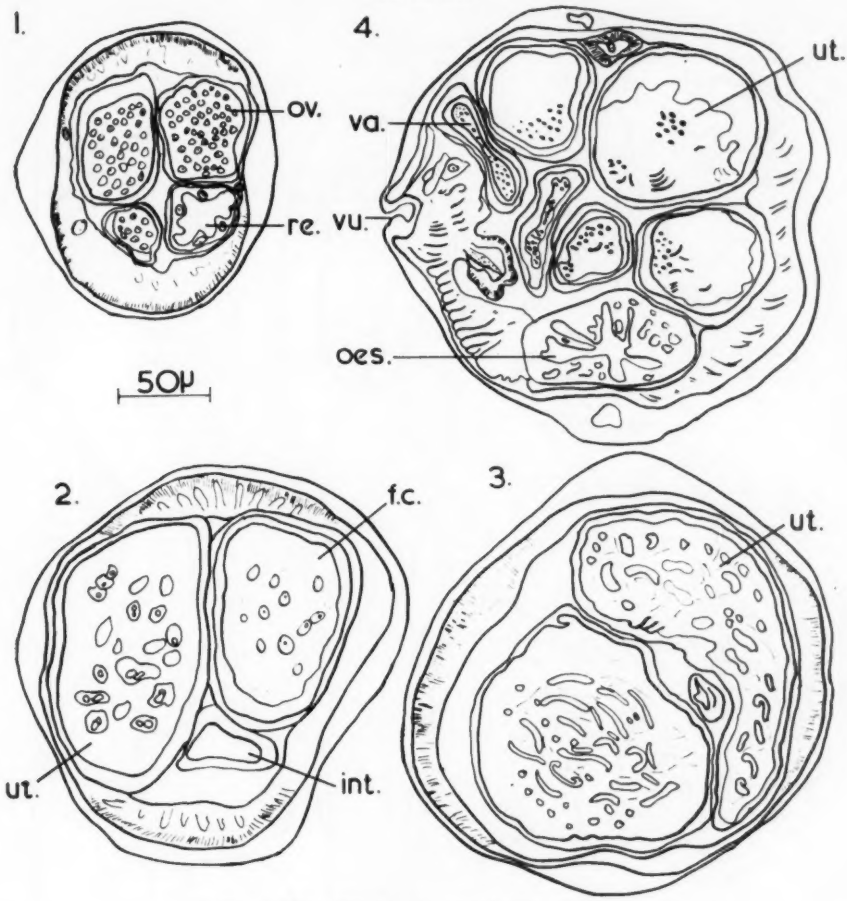

PLATE III. The female reproductive system.

FIGURE 1. Transverse section showing posterior coils of ovary and rectum.
FIGURE 2. Transverse section showing posterior part of uterus acting as fertilization chamber.
FIGURE 3. Transverse section showing paired uteri.
FIGURE 4. Transverse section through vulva.

PLATE II. Spermatogenesis.

FIGURE 1. Multiplication zone of testis.
FIGURE 2. Spermatogonia.
FIGURE 3. Mature spermatogonia showing paired chromosomes.
FIGURE 4. First reduction division.
FIGURE 4. Second reduction division.
FIGURE 6. Cytoplasmic reduction of spermatozoa.
FIGURE 7. Mature spermatozoa showing lining out of chromosomes.

PLATE IV

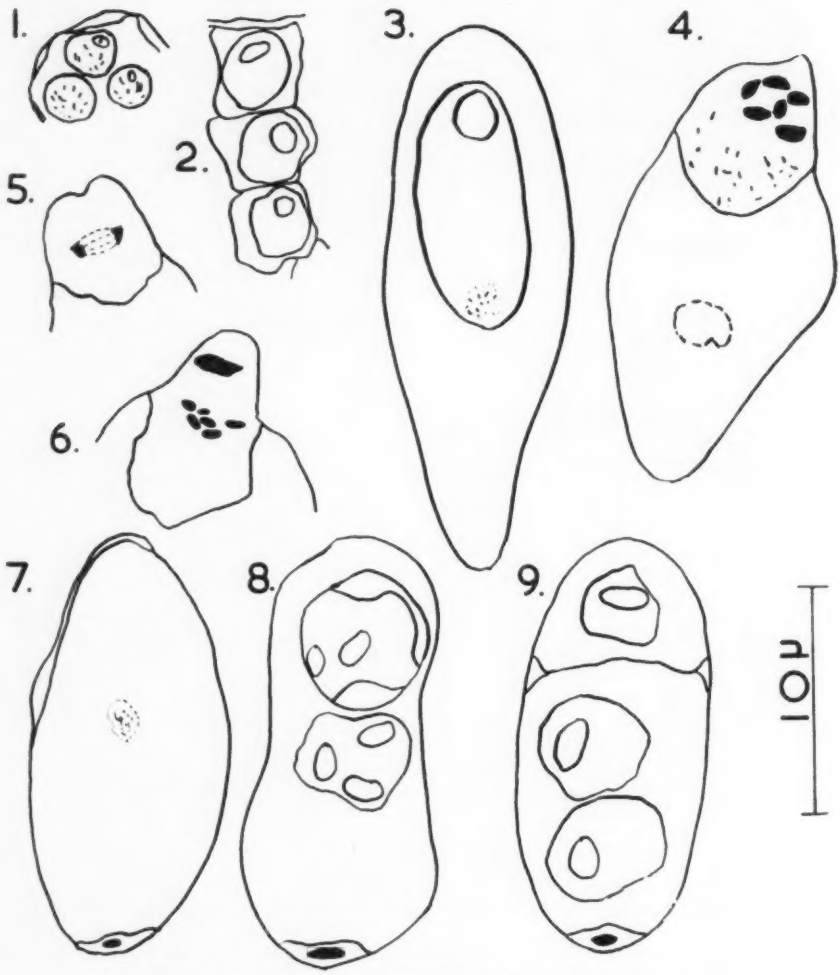

PLATE IV. Oogenesis, fertilization and early development.

FIGURE 1. Scattered nuclei in anterior region of ovary.
FIGURE 2. Cubical oogonia.
FIGURE 3. Primary oocyte.
FIGURE 4. Metaphase plate of first reduction division.
FIGURE 5. Telophase showing spindle.
FIGURE 6. Formation of first polar body.
FIGURE 7. Zygote.
FIGURE 8. Early cleavage of embryo.
FIGURE 9. Early cleavage of embryo.

PLATE V

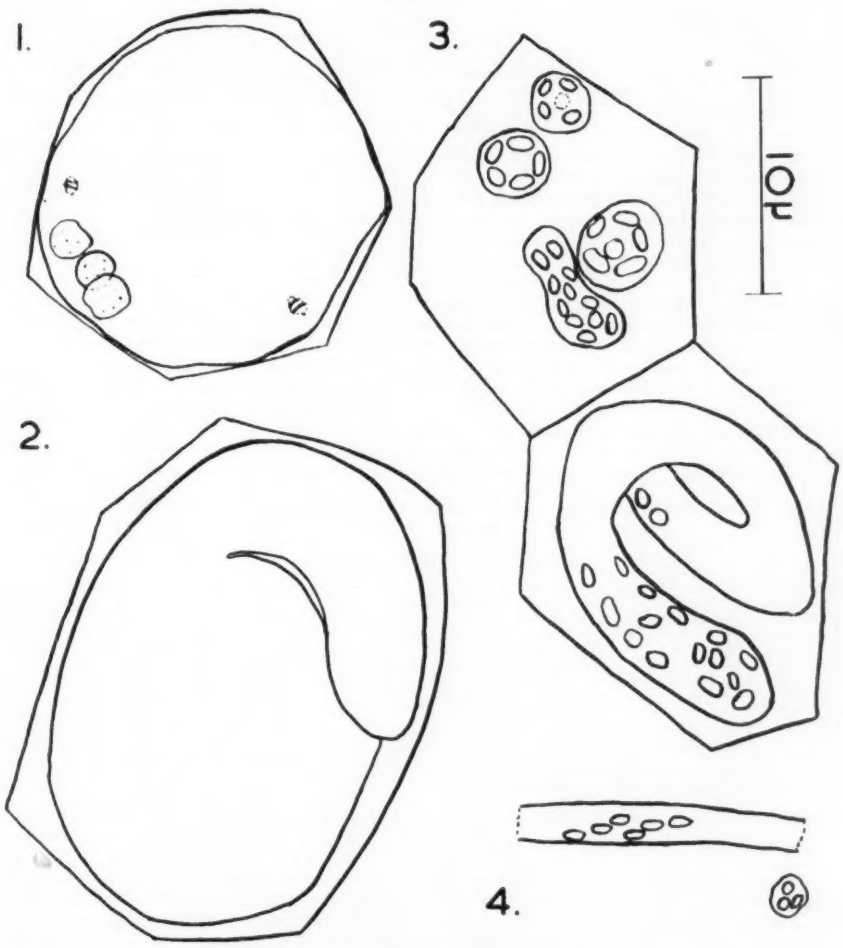

PLATE V. Development of microfilaria.

FIGURE 1. Blastula.
FIGURE 2. Typical gastrula.
FIGURE 3. Young coiled microfilaria.
FIGURE 4. Young microfilaria free from membranes.

RESEARCH NOTE

FIELD OBSERVATIONS ON THE MIGRATION OF MARKED *AUSTRALORBIS GLABRATUS* SNAILS.

The movements of marked *Australorbis glabratus* snails released in a Puerto Rican stream were observed by Pimentel et al. (1957, Am. J. Trop. Med. Hyg. **6**: 576–580). Since these authors reported that the marking lacquer they used wore off and changed color after 2 weeks, a search was made for long-lasting, quick-drying, non-toxic substance for use in the present study. These characteristics were found in "deco-write" paints (Ball Point, Craf-Tube, by the Craftint Manufacturing Company, Cleveland 10, Ohio).

Snails 10 mm or larger were prepared for marking by cleaning small areas of the ultimate whorl with a soft cloth. Paints of several bright colors were then applied to the clean surfaces with a ball point. Eighteen groups of marked snails, ranging from 10 to 600 per group, were released on different days in a staked 25-foot section of the El Toro Creek, Bayamón, Puerto Rico, flowing 1 to 2 miles per hour through a heavy growth of "elephant ear" plants (*Caladium* sp.). Collections of snails were made beginning after 1 hour and up to 92 days from an area 300 feet upstream and 200 feet downstream from the release point.

The majority of snails recovered was collected during the first 7 days after release. Twenty-seven percent of the retrieved living snails were found upstream as compared to only 3 percent downstream (table I). No marked snails were collected + 200 feet above or – 100 feet

TABLE 1. *Periodic recovery of marked snails at distances upstream and downstream from point of release.*

Days between release and collection	Distance in feet				Percent of total marked snails recovered	
	Upstream		Release point	Downstream		
	+ 200 to + 101	+ 100 to + 0	25	– 0 to – 100	Living	Dead
1/12	0	—	113 (3)*	0	16.1	0.4
1– 7	0	30 (11)*	144 (190)	6 (6)*	6.5	7.5
8–21	—	13 (3)	48 (59)	1 (6)	3.5	3.8
22–35	7	53 (2)	33 (19)	8 (16)	5.4	2.0
36–49	1	15 (4)	5 (1)	0 (2)	2.7	0.9
50–63	3	23 (1)	0 (1)	0	2.5	0.2
64–77	0	2	0	0	0.9	0.0
(Total)	11	136 (21)	343 (273)	15 (30)	16.4	10.5

* Figures in parentheses indicate dead snails.

below from the site of introduction; likewise, marked snails were not recovered after 77 days from the time of releasing. Some dead snails were recovered, most of them from the area of release.

A predominantly downstream movement of *A. glabratus* snails was observed by Pimentel et al. (loc. cit.), and was attributed to the force of the water. Our data are at variance with those of the above authors. MYRON G. RADKE AND LAWRENCE S. RITCHIE, *Department of Medical Zoology, Walter Reed Army Institute of Research; Field Team, U. S. Army Tropical Research Medical Laboratory, San Juan, P. R.*

A KNOWN AND A NEW FILARIID FROM INDIAN BIRDS

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DIPLOTRIAENIDAE Anderson, 1958

APROCTINAE Yorke and Maplestone, 1926

Aprocta Linstow, 1883

Aprocta turgida Stossich, 1902

One male and three female specimens of this species were collected from the nasal cavity of the Indian Oriole, *Oriolus oriolus kundoo*. Stossich (1902) described this species for worms found in *Larus argentatus*. The present material represent the first record of this species from India and also from a new host. Measurements in millimeters recorded by the writer follow.

Male: Length, 15.87; maximum width, 0.52; distance from head end to nerve ring, 0.38; to exeretory pore, 0.46; length of oesophagus, 0.75; tail, 0.15; spicules, 0.27.

Female: Length 34.27 to 36.41; maximum diameter, 0.73 to 0.76; distance from head end to nerve ring, 0.42 to 0.44; to exeretory pore, 0.48 to 0.52; length of oesophagus, 0.98; anus opening at the tip of the tail; anterior end to vulva, 0.14 to 0.15; eggs embryonated, 0.048 to 0.052 by 0.029 to 0.033.

Host: *Oriolus oriolus kundoo.*

Habitat: Nasal Cavity.

Locality: Hyderabad, India.

Specimens: Museum, Zoology Department, Osmania University.

ONCHOCERCIDAE Chabaud and Anderson, 1959

ORNITHOFILARIINAE Chabaud and Anderson, 1959

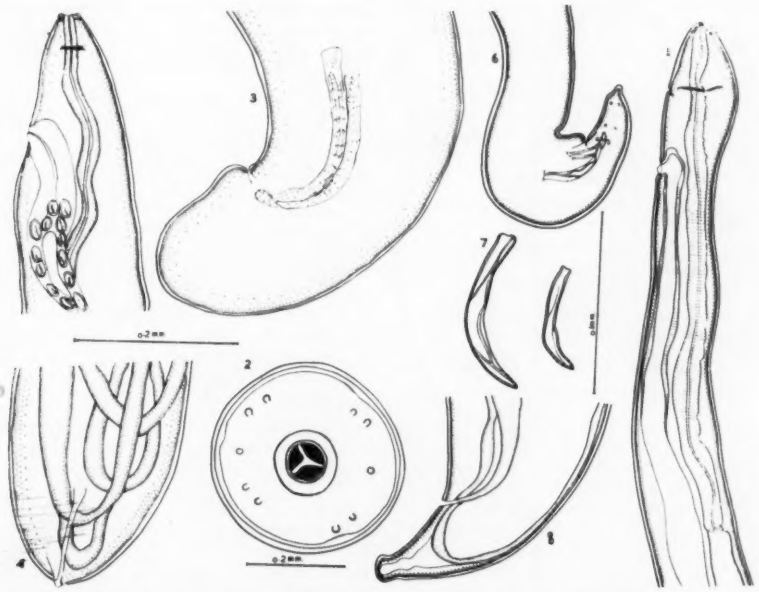

FIGURE 1. Comparison of *Aprocta turgida* Stossich, 1902, and *Aproctoides alii*. *Aprocta turgida* Stossich, 1902 (after Skrjabin, 1917): 1, anterior end, lateral view; 2, end-on view; 3, posterior end, lateral view; 4, female tail end. *Aproctoides alii:* 5, female, anterior end; 6, male posterior end; 7, spicules; 8, female, tail end. Scales: 0.2 mm (larger) applies to figures 1, 3, 4 to 6, and 8; 0.1 mm applies to figure 7; 0.2 mm (smaller) applies to figure 2.

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Aproctoides Chandler, 1929

Aproctoides alii n. sp.

A single male and two females of this species were collected from the orbital cavity of the Southern Green Pigeon, *Crocopus phoenicopterus*. Measurements are in millimeters.

Diagnosis: Worms of medium size with having extremities slightly tapering. Male, half as long as female with spirally twisted tail. Cuticle thin and without transverse striae. Two pairs of submedian cephalic papillae and a pair of lateral amphids. Nerve ring surrounds anterior part of oesophagus 0.07 and 0.09 from head end in male and female, respectively. Excretory pore opens immediately behind nerve ring.

Oesophagus divided into very short muscular and very long glandular portion measuring, respectively, 0.07 and 0.48 in male and 0.08 to 0.12 and 0.52 to 0.60 in female; entire oesophagus 0.55 and 0.60 to 0.70 long in respective sexes, roughly occupying one-eighteenth and one-thirtieth of body length in male and female, respectively.

Male: 9.57 long, with a maximum diameter of 0.12; tail spirally coiled, measuring 0.089 in length, with three pairs of small sessile papillae somewhat ventral in position. Neither preanal nor circumanal papillae present. Tail devoid of caudal alae. Spicules short and lightly chitinized, measuring 0.062 and 0.097 in length, respectively; similar in shape with slightly expanded heads and pointed tips.

Female: 18.21 to 19.3 in length and 0.15 to 0.18 in maximum diameter. Tail digitiform, with rounded tip; cloaca 0.089 from posterior end, Vulva flush with body opening in oesophageal region about 0.19 to 0.26 from head end; vagina directs posteriorly from vulva; two ovaries in posterior quarter of body.

Host: Crocopus phoenicopterus.

Habitat: Orbital cavity.

Locality: Hyderabad, India.

Type specimens: Museum, Zoology Department, Osmania University.

Discussion. Chandler (1929) erected the genus *Aproctoides* for worms collected from the orbital cavity of the magpie robin, *Copsychus saularis;* the second species was reported by Ali in 1956 from *Francolinus pictus;* the present worm constitutes the third species for the genus. The following table indicates various measurements and differences among the three species. All measurements in millimeters.

	A. lissum Chandler, 1929	*A. papillatus* Ali, 1956	*Present species*
Length male	12	19.25 to 26.8	9.57
Oesophagus	0.72	1.15 to 1.25	0.55
Tail	0.14	0.1 to 0.12	0.08
Spicules	0.3 and 0.13	0.31 and 0.14	0.062 and 0.09
Papillae	absent	2 pairs	3 pairs
Length female	unknown	41.28	18.21 to 19.3
Oesophagus	—	2.0	0.60 to 0.70
Tail	—	0.06	0.10
Vulva from head end	—	1.8	0.19 to 0.26

In view of the differences in body length, proportionate length of oesophagus, spicules, number of caudal papillae, and position of the vulva, the present species is regarded as new. It is proposed to name it *Aproctoides alii* n. sp. after Dr. S. Mehdi Ali of Osmania University, Hyderabad, India.

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AMEERIA SULTANAE N. G., N. SP., FROM THE COMMON GREY HORNBILL *TOCKUS BIROSTRIS*, IN INDIA

SYED MEHDI ALI

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ONCHOCERCIDAE (Leiper 1911 sub family) Chabaud and Anderson, 1959.
ORNITHOFILARIINAE Chabaud and Anderson, 1959.
Ameeria, n. g.
Ameeria sultanae n. g., n. sp.

Two male and six female specimens of this filarioid fere recovered from the common grey hornbill, *Tockus birostris*. The worms were found among the muscles of the orbital cavity and also underneath the skin, alongside the trachea.

Diagnosis: Body of worm straight, having a milky white color, and of uniform diameter; rounded at extremities with head end exhibiting a slightly swollen appearance and tail being inwardly curved in both sexes. Cuticle thin with deeper longitudinal lines reinforced with superficial transverse striae; superficial striations of two types; deeper striae spaced equidistant with 5 to 7 still finer and more superficial striae closely arranged between two consecutive-deeper ones; presence of rugae appears like smaller bosses at lateral fields.

Mouth terminal and surrounded by four pairs of cephalic papillae arranged in two circles, and a pair of laterally situated amphids. Nerve ring surrounds oesophagus at a distance of 0.09 to 0.11 mm from anterior end in male and 0.12 to 0.14 mm in female. Exretory pore located at 0.11 and 0.14 mm from head end in two sexes, respectively. Oesophagus very short and undivided measuring 0.18 to 0.20 mm long in male and 0.19 to 0.21 mm in female.

Male: Less than half as long as female measuring 11.38 to 12.10 mm in length and 0.18 to 0.28 mm in maximum width; tail very short, 0.048 to 0.058 mm long and devoid of alae, but provided with five pairs of sessile but sufficiently prominent papillae: two pairs pre-anal, one adanal and two postanal. Spicules unequal and dissimilar, left 0.82 to 0.91 mm long and right 0.16 to 0.18 mm, former almost five times as long as latter; left spicule with an oval tip, and conical elevation short distance behind tip; right spicule of almost uniform thickness except somewhat expanded near tip, and with spur-like projection, just behind tip.

Female: Almost double size of male, measuring 28.17 to 31.92 mm in length and 0.36 to 0.40 mm in width. Vulva flush with body located 0.60 to 0.73 mm from head end. Vagina directed posteriorly for short distance and then forwards uniting with uteri which extend almost to base of oesophagus than taking a backward course, running to tail region. Microfilaria stumpy and measure 72 microns long and 7 microns wide. Tail very short as in male, being 0.063 mm long.

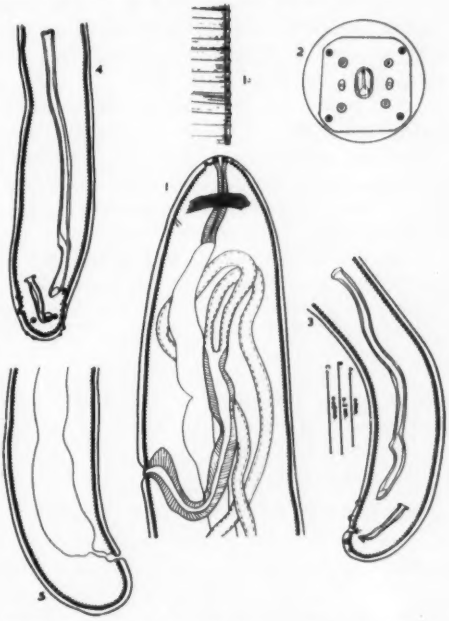

FIGURE 1. *Ameeria sultanae* n. g., n. sp. 1, female, anterior end, lateral view (scale B); 1a, female; rugae and striae (scale B); 2, female, head end-on view (scale C); 3, male, posterior end, lateral view (scale A); 4, male, posterior end, ventral view (scale A); 5, female, posterior end, lateral view (scale B).

Host: *Tockus birostris.*
Habitat: Orbital cavity.
Locality: Hyderabad, India.
Type specimens: Museum, Zoology Department, Osmania University.

Discussion. In spite of possessing similari-

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ties of characters with *Paronchocerca* Peters, 1936, and *Paraprocta* Maplestone, 1931, the present worm cannot be assigned to either of these genera. The shape of spicules, the presence of cuticular rugae, the post-oesophageal vulva are the characters similar to those found in *Paronchocerca*. In having an undivided oesophagus, in the absence of the rod-like thickenings of the cuticle, and the anogenital papillae, in possessing four pairs of cephalic papillae (as against two pairs in *Paronchocerca*) and in the absence of terminal caudal papillae on the tail in both the sexes, it differs from the said genus. The undivided oesophagus, post-oesophageal vulva, and the unequal spicules are characters in which the present worm shares with *Paraprocta*. According to Maplestone (1931) the caudal papillae are absent, but Chandler reports inconspicuous papillae—one preanal one postanal pair.

In the present worm there are five pairs of fairly well developed caudal papillae. In *Paraprocta* the anus is reported as closed. The tail in both sexes of the present specimens is very short, but comparatively longer than *Paraprocta*.

The length of the body and of spicules, in millimeters, are as follows in the two worms:

	Paraprocta		The Present Worm
	Maplestone	Chandler	
Right spicule	0.065	0.06 to 0.07	0.16 to 0.18
Left spicule	0.105	0.18 to 0.21	0.82 to 0.92
Body, male	7.5 to 10.5		11.38 to 12.10
female	16 to 18		28.17 to 31.92

In view of the characters observed and the differences exhibited by the worms, it is thought desirable to erect a new genus for the species. This species seems to belong to a genus intermediate between *Paraprocta* and *Paronchocerca*. It is proposed to name the new genus *Ameeria* after my student Ameer Sultana who had so kindly placed the material at my disposal.

The assignment of this genus to Dipetalonematinea Wehr, 1935, seems to be more appropriate rather than its place under Ornithofilariinae Chabaud and Anderson, 1959, because of the latter subfamily having been described as "possessing circumanal papillae," which are not present in *Ameeria*. If Chabaud and Anderson's (1959) assignment of *Paraprocta* and *Paronchocerca* to Ornithofilariinae is to be accepted, then the above mentioned character needs revision.

Generic diagnosis: Head with eight cephalic papillae and a pair of amphids. Cuticle striated and reinforced with rugae. Oesophagus short and undivided. Vulva post-oesophageal. Spicules very unequal and dissimilar. Caudal papillae, five pairs. Tail very short in both sexes. Viviparous.

Type species: Ameeria sultanae.

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TWO NEW SPECIES OF *LEMDANA* SEURAT, 1917, FROM INDIAN BIRDS

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Hyderabad, India

ONCHOCERCIDAE Chabaud and Anderson, 1959
LEMDANINAE Lopez-Neyra, 1956
Lemdana Seurat, 1917

This genus was established by Seurat, 1917, with *L. marthae* as its type species. Since then eight more species have been added. These are *L. micropenis* Travassos, 1925; *L. behningi* Lewaschoff, 1929; *L. limboonkengi* Hoeppli and Hsu, 1929; *L. lomonti* Desportes, 1947; *L. corvicola* Shikhobalova, 1948; *L. urbaini* Campana-Rouget, 1949; *L. skrjabini* Kasimov, 1952; *L. subspiralis* (Diesing, 1851), Lopez-Neyra, 1956; and *L. dartevelli* Ezzat and Tendros, 1958. Chabaud and Choquet (1955) regard *L. urbaini* as identical with *Pharyngosetaria ardeae,* hence it is excluded from the key to species given at the end. The last two species could not be included in the key due to inaccessibility to literature.

Two new species are being described in this communication.

Lemdana alii n. sp.

Three males and one female were collected from underneath the skin of the neck of the pale harrier, *Circus macrourus.*

Diagnosis: Worms thin, having straight body of milky white colour when alive, with slightly dome shaped anterior extremity and bluntly rounded tail in both sexes. Cuticle thin almost transparent, with faint transverse striae observed under higher magnification. Mouth devoid of lips, but surrounded by a pair of cephalic papillae on either side; cervical papillae not observed. Nerve ring and exretory pore 0.09 to 0.10 mm and 0.13 to 0.15 mm respectively, from head end in male and 0.12 mm and 0.16 mm in female. Oesophagus somewhat dilated anteriorly divided into a very short muscular and a long posterior portion. Muscular portion measures 0.18 to 0.20 mm in length in male and 0.28 mm in female and glandular portion 0.42 to 0.58 mm and 0.67 mm long in respective sexes.

Male: Slightly smaller than female, being 7.45 to 8.2 mm in length and 0.15 to 0.18 mm in maximum thickness; head diameter 0.08 to 0.1 mm. Testis extend into oesophageal region, reflexes a little posterior to nerve ring and runs backwards to junction of oesophagus and intestine to terminate in posteriorly directed tip. Cloacal aperture almost subterminal being 0.017 to 0.019 mm. from tip of tail. Caudal papillae five, two just in front of cloaca, one pair slightly post cloacal and solitary papillae at tip. Spicules dissimilar and unequal slightly chitinized, measuring 0.26 to 0.32 mm and 0.012 to 0.015 mm in length, with expanded heads and pointed tips and bulge in middle portion.

Female: Thicker than male, measuring 9.86 mm in length, and with maximum diameter of 0.28 mm. Anterior ovary short distance behind vulva posterior with tip right in vinicity of tip of tail. Vulval opening surrounded by lip-like structures and followed by a fairly muscular vagina measuring 0.38 mm in length. Distance between head end and vulva 0.30 mm. Tail very short, being 0.029 mm long

Host: *Circus macrourus.*
Habitat: Subcutaneous connective tissue.
Locality: Hyderabad, India.
Type specimens: Museum, Zoology Department, Osmania University.

Discussion: This worm differs from all the known species of the genus in being the smallest and the thinnest. Both the type species and *L. micropenis* have males about 10 to 12 mm long. slightly longer than the males of the present worm (7.4 to 7.48 mm), but the females of the former two, are three and five times, respectively, longer than those of the worm under discussion. The measurements of the spicules, number of the caudal papillae, and the position of the vulva differ in all three. Hence, the present worm is regarded as a new species and it is proposed to name it *Lemdana alii* after Dr. Syed Mehdi Ali of Osmania University, India.

Lemdana singhi n. sp.

A single pair of this species was recovered from under the skin of the neck of the black-headed cuckoo shrike, *Lalage sykesii.*

Diagnosis: Worms very thin bodied with a marked difference in their lengths; males measure 4 mm, females 16.41 mm long. Maximum diameter of body 0.06 mm in male and 0.09 mm in female. Anterior and posterior extremities bluntly rounded with body of uniform diameter. Cuticle thin, transparent, and almost devoid of transverse striae. Cephalic papillae four in number; amphids

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present, lateral in position. Nerve ring surrounds muscular portion of oesophagus at a distance of 0.04 mm from head end in male and 0.05 mm in female. Excretory pore in vicinity of junction of anterior and posterior portions of oesophagus. Muscular portion of oesophagus very short measuring 0.06 mm in length in male and 0.08 mm in female. Glandular portion very long and measures 0.6 mm and 0.8 mm in male and female, respectively.

Type specimens: Museum, Zoology Department, Osmania University.

Discussion. In possessing the smallest male of the known species of the genus, this worm differs from all. The female has almost the same length as that of *Lemdana corvicola.* In the absence of the male of *L. corvicola,* it is not possible to compare the male characters of the two.

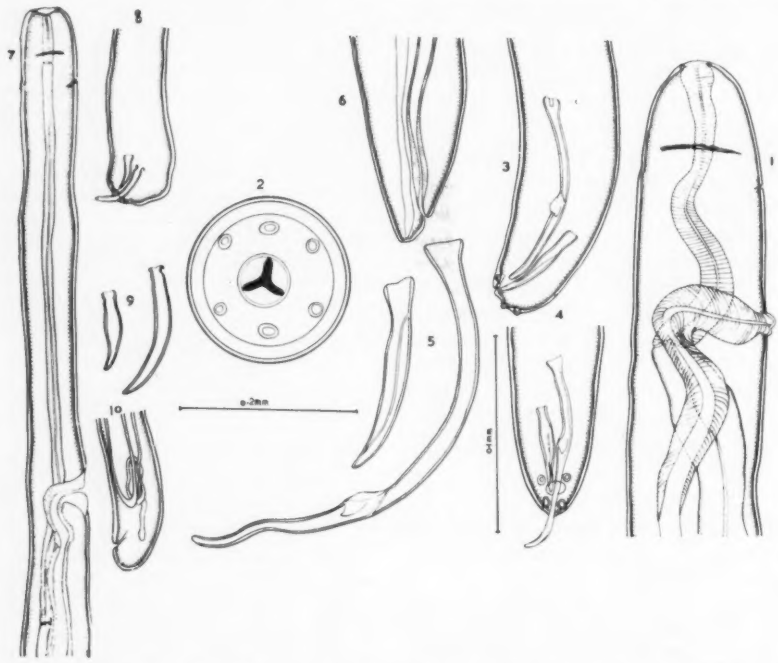

FIGURE 1. Comparison of *Lemdana alii* and *L. singhi. Lemdana alii:* 1 female anterior end (scale 0.2 mm); 2, end-on view (scale 0.1 mm); 3, male tail, lateral view (scale 0.2 mm); 4, male tail, ventral view (scale 0.2 mm); 5, spicules (scale 0.1 mm); 6, female tail, lateral view (scale 0.2 mm). *Lemdana singhi:* 7, female anterior end (scale 0.2 mm); 8, male posterior end lateral view (scale 0.2 mm); 9, spicules (scale 0.1 mm); 10, female tail, lateral view (scale 0.2 mm).

Males: Cloaca almost subterminal. A pair of precloacal and another pair of post-cloacal papillae present. Spicules very small and lightly chitinized measuring 0.064 mm and 0.039 mm.

Female: Four times longer than male, vulva in posterior region of glandular oesophagus, 0.58 mm from head end and leading into a muscular vagina measuring 0.26 mm in length. Ovaries two, located at anterior and posterior third of body; posterior ovary extends up a short distance in front of subterminal cloaca, 0.026 mm from tip of tail.

Host: Lalage sykesii.

Habitat: Subcutaneous connective tissue.

Locality: Hyderabad, India.

However *L. corvicola* has an oesophagus 0.27 to 0.34 mm long as against 0.88 mm for that of the present worm. In *L. corvicola* the tail is 0.36 to 0.43 mm long and possesses a bifid spike at its tip, whereas in the present worm the tail is very short, the anus being just 0.02 mm from its tip, which is devoid of either papillae or spike. In view of the characters discussed above, the present species is regarded as new. It is named *Lemdana singhi* n. sp. after Professor S. N. Singh of Osmania University.

Key to Species of Lemdana *Seurat, 1917*

Males unknown 1
Males known 3

1. Females 16 to 18 mm long; oesophagus 0.29 to 0.34 mm; tail 0.36 to 0.43 mm
L. corvicola Shikhobalova, 1948
Females more than 30 mm long 2

2. Females 32 to 33 mm didelphic vulva 2 to 3 mm from head end
L. Limboonkengi Hoeppli and Hsü, 1929
Females 37 mm tridelphic, vulva 0.56 mm from head end ..... *L. lomonti* Desportes, 1947

3. Males more than 20 mm long 4
Males less than 20 mm long 5

4. Males 26 mm; females 69 to 94 mm; spicule ratio 7:1 (L.S. 217 mm; R.S. 0.33 mm) caudal papillae: 4 pairs peranals, 2 pairs postanal ..... *L. behningi* Lewaschoff, 1929
Males 29.5 mm; females 42.9 to 52.2 mm, spicule ratio 2:1 (L.S. 0.29 mm; R.S. 16 mm) caudal papillae: two indistinct
L. skrjabini Kasimov, 1952

5. Males 10 to 14 mm 6
Males much smaller 7

6. Females 34 mm; spicule ratio 10: 15; caudal papillae: four circumanal and two postanal
L. marthae Seurat, 1917
Females 45 to 46 mm; spicules almost equal (L.S. 0.088; R.S. 0.080 mm)
L. micropenis Travassos, 1925

7. Males 7.4 to 8.2 mm; females 8.9 mm; left spicule 0.26 to 0.32 mm; right spicule 0.12 to 0.15 mm; caudal papillae: one pair preanal, one pair postanal and one papillae at the tip ..... *L. alii* n. sp.
Males 4 mm; females 16 mm; L.S. 0.064 mm; R.S. 0.039 mm; caudal papillae; one pair preanal one pair postanal
L. singhi n. sp.

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RESEARCH NOTE

INFLUENCE OF TRICHINOSIS ON *SCHISTOSOMA MANSONI* IN MICE.

A wide variety of *Schistosoma mansoni* antigens serologically react with antibodies to *Trichinella spiralis* infections (Liu and Bang, 1950, *Proc. Soc. Exp. Biol. Med.* **74**: 68–72; Senterfit, 1958, *Am. J. Hyg.* **68**: 148–155; Anderson, 1960, *Am. J. Trop. Med. Hyg.* **9**: 299–303). In 1959–60, experiments were undertaken at the U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico, to determine whether infection with *T. spiralis* would influence the worm burden from a superimposed infection with *S. mansoni*.

Mice (Bagg-Swiss strain) were infected via stomach tube with 125 larvae of *T. spiralis*. When this infection was 5 weeks old, half of the animals plus an equal number of uninfected mice of the same sex and age were exposed, by tail immersion, to 50 or 200 cercariae of *S. masoni*. Six weeks following this exposure all mice were sacrificed and their worm burdens determined. Schistosomes were recovered by perfusion and trichina larvae by digestion of entire eviscerated carcasses.

In the first experiment, female mice were exposed to 50 cercariae. The mean number of schistosomes recovered at autopsy was 14 ± 5 from 20 mice previously infected with *T. spiralis* and 15 ± 7 from 18 control animals. The difference (1 ± 2 worms) was not significant. When this experiment was repeated with male mice, the mean number of schistosomes recovered was 20 ± 5 from 18 mice with *T. spiralis* versus 16 ± 6 from 20 control animals. The difference (4 ± 2 worms) is of questionable significance. Thus, prior infection with 125 trichina larvae did not influence materially the numbers of schistosomes developing from 50 cercariae.

In the third experiment female mice were exposed to 200 cercariae. The mean schistosome recovery was 30 ± 23 worms from 19 mice in the test group compared to 55 ± 15 worms from 19 mice in the control group. The difference (25 ± 6 worms) was significant. While prior trichina infections did not reduce the numbers of schistosomes developing from moderately light infections (50 cercariae), they did reduce the numbers developing from heavy infections (200 cercariae).

The double infection reduces the numbers of schistosomes, but did not influence the numbers of trichina larvae. The mean *T. spiralis* burden in 19 test mice was 1080 ± 1140 larvae compared with 950 ± 540 in 16 control mice. The difference (130 ± 230 larvae) was not significant.—L. A. JACHOWSKI, JR., *Naval Medical Research Institute, Bethesda Md.* AND G. A. BINGHAM, *National Institutes of Health, Bethesda, Md.*

RESEARCH NOTE

A SAMPLING TECHNIQUE FOR INTRANASAL CHIGGERS (TROMBICULIDAE).

From June through August 1956, I examined over 1,500 small wild mammals in East Africa for parasitic Acarina. Intranasal mites were recovered from many of these animals, but because of the need to retain undamaged skulls for host identification and the time required to expose nasal passages and search for these secretive parasites, numerous skulls remained uninvestigated. During the latter part of this survey, the following simple technique for rapid examination for nasal mites, without destruction of skulls, was devised.

The method consists of forceful washing of the nasal passages with water, followed by collection and examination of the washings. The tip of a 20-gauge needle is cut off 2 mm from its base. The stub is ground to a smooth, blunt tip. The dead animal is grasped firmly by the throat in order to close the trachea and esophagus. The needle, attached to a 5-cc syringe containing water, is introduced into one of the nostrils. The water, forcefully flushed through the nasal passages, is collected as it emerges from the other nostril and mouth. The procedure is repeated 2 or 3 times alternately in either nostril. Intranasal chiggers, if present, will generally be found in the sediment along with mucous strands and, occasionally, blood. Epidermoptid and speleognathid mites may also be recovered by this method. The latter, being extremely hydrophobic, are nearly always found floating on the surface of the washings.

The technique has subsequently been used in Egypt and Panama to recover intranasal mites from over 60 animals, including rodents, bats, edentates, and a bird. Many more mammals were uninfested; negative findings were confirmed by exposing and examining their nasal passages. Negative washings, however, do not necessarily exclude the possibility of infestation, and this technique should never replace visual examination of nasal passages whenever the latter is feasible in terms of time and expendability of hosts. Mite specimens are not always flushed into the collecting dish. Some become lodged in the turbinal folds and mouth. It is possible that certain intranasal chiggers may attach firmly to the nasal mucosa in the same manner that their ectoparasitic counterparts attach to skin. However, I have never seen intranasal chiggers attached firmly. Audy and Nadchatram (1957, Malaysian Parasites **26**: 187–230), referring to *Traubacarus* spp. in the nasal passages of Malaysian rats, state: "The larvae always appear to lie in the mucus and we have never found one attached so that any effort is required to remove it." The nasal washing technique, therefore, offers a rapid means for large-scale assay of intranasal chiggers without damage to the host skull. It may also provide a means for garnering data on host-parasite relationships and infestation rates. Dr. J. R. Audy (personal communication) has commented that this method, if used carefully on lightly anesthetized animals, would allow the collection of intranasal mites from hosts in mark-release-recapture experiments. In addition, the possibility of recovering intranasal mites from alcohol-preserved museum specimens by this method has been suggested by Dr. A. Fain (personal communication).

This note is a contribution of the Scientific Working Party on Ectoparasites sponsored by the U. S. Naval Medical Research Unit No. 3, Cairo, Egypt, and the East African Veterinary Research Organization, Muguga, Kenya, 1956. I was aided by Office of Naval Research Grant Nonr 1809(00) to the Department of Zoology, University of Maryland, College Park.—CONRAD E. YUNKER, *U. S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Middle America Research Unit, Balboa Heights, Canal Zone, and Rocky Mountain Laboratory, Hamilton, Montana.*

EXPERIMENTAL TRICHINOSIS IN THE GOLDEN HAMSTER. IV. SOME OBSERVATIONS ON THE LEUKOCYTIC RESPONSE *

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The discovery by Brown (1898) that eosinophilia is a rather constant clinical symptom of *Trichinella spiralis* infection was followed by reports of eosinophilia in certain animals experimentally infected with this parasite. Among rodents, for example, the infected guinea pig shows a marked eosinophilia (Opie, 1904; Hamann, 1943), but there is a lesser response in the rat (Williams and Bentz, 1903; van Someren, 1938; Beahm and Downs, 1939; Hamann, loc. cit.; and Larsh and Nicholls, 1949). Although the latter authors failed to demonstrate eosinophilia in the infected mouse, Stein (1949) reported 4 to 20 percent eosinophilia and used the depletion of eosinophils as a bioassay for adrenocortical activity. The striking change in eosinophil numbers overshadows other leukocytic changes, but these too generally occur following infection.

MATERIALS AND METHODS

Two groups of golden hamsters (*Mesocricetus auratus*) were used. The animals in each group received a dose of 225 ± 10 *Trichinella* larvae isolated as described previously (Bernard, 1961). Group I consisted of 5 male and 5 female animals, 8 to 10 weeks old and weighing from 85 to 100 g at time of infection. Four of each sex were infected; the other two animals served as controls. Blood was drawn from these animals just before infection and on days 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 30, 33, 39, 42, 45, 48, 52, 56, 60, 65, 70, 75, 85, and 103 post infection (PI). For blood collection the animals were lightly anaesthetized with sodium pentobarbital (7.2 mg per 100 g body weight), the hairs of the tail clipped, the dorsal surface moistened with 70 percent ethanol, allowed to dry, and then deeply incised transversely with a sterile blade. Smears were made and stained with freshly prepared Wright's stain. Two to three hundred leukocytes were counted from each smear.

Group II consisted of 201 animals, 154 infected (at 8 to 10 weeks of age) and 47 control. About half were male. The animals of this group were bled by the same procedure, but only once, at times ranging from 7 to 300 days PI. These animals were being used in other experiments and were sacrificed just after the blood was taken. Total leukocyte and total eosinophil counts were performed on these blood samples. The eosinophil counting technique of Speirs and Meyer (1949) was used.

In order to minimize the effects of any variations in the physiological or morphological state of the blood elements brought about by activity, lighting, etc. (Carnicero, 1944; Halberg and Visscher, 1950; Halberg et al, 1953; and Halberg and Chaudry, 1960) all animals were bled between 7:00 and 11:00 P.M., the most active period in that hamster colony (Bernard, 1959). Animals were housed in individual cages in an animal room and handled only for routine weighing. The room's temperature was thermostatically controlled at 23 C. The room was illuminated by sunlight (and artificial light seldom later than 5:00 P.M.). Purina Lab Chow pellets and tap water were available ad libitum.

RESULTS

Group I. Alterations of the differential counts are summarized in figures 1 through 10. The populations of cells bearing eosinophilic granules were affected by the presence of *Trichinella* in the host. The number of circulating eosinophils began to increase about day 13 PI, reached a peak from 16 to 19 days PI, and returned to "normal" about a week later. The magnitude of change in numbers of eosinophils is not correlated with the severity of the infection as revealed by body weight losses. For example, animals T1, T2, and T7 lost the greatest percentages of weight (23 to 27 percent) while they ranked 5th, 3d, and 4th, respectively, in eosinophil response. The greatest eosinophilia was found in T3 which lost 14 percent of its initial weight. T4 and T6 lost no weight yet had eosinophil percentages of 12 and 14 respectively.

In the normal hamster lymphocytes outnumber the neutrophilic leukocytes approximately 2 to 1. Four to 12 days PI this relationship was altered so that the neutrophils became dominant. It would appear that a second

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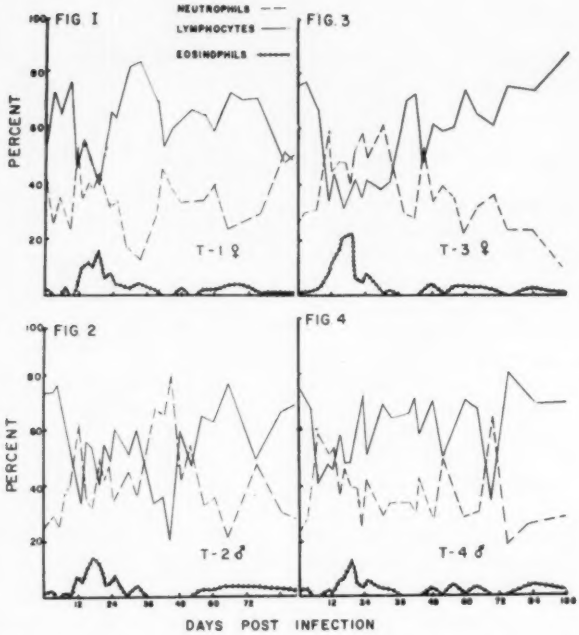


FIGURES 1–4. Alterations in the differential blood counts of four infected golden hamsters of Group I.

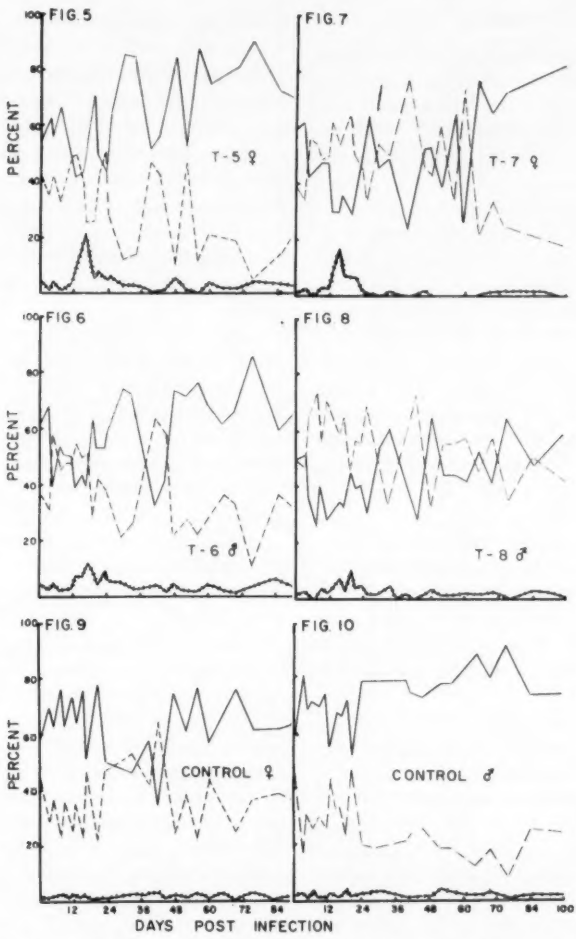
decrease in the number of circulating lymphocytes might have occurred about 40 days PI, but a similar reversal was also observed in one of the control animals. The neutrophils remained the dominant leukocytic type for varying periods. They returned to within the normal range as early as day 20 or as late as day 70 PI. Again no correlation between the intensity of the infection as indicated by weight loss and the disturbance of the lymphocyte-neutrophil relationship was observed. I have never observed cells recognizable as "monocytes" in normal or infected golden hamsters.

TABLE I. *Absolute leukocyte and eosinophil counts of normal and* Trichinella-*infected golden hamsters of Group II.* (c, control.)

Days post infection	No. of animals	Leukocytes Mean	Leukocytes Range	Eosinophils Mean	Eosinophils Range	Mean percent eosinophils
7	9	5260	3100– 7600	40	0– 85	0.8
7 (c)	10	4425	1550– 7500	40	15–125	0.9
14	9	4980	3700– 6650	148	65–205	3.0
21	9	6540	2300–14200	85	0–180	1.3
28	8	5575	2650– 8750	76	30–155	1.4
28 (c)	8	5140	3950– 5950	42	5–150	0.8
35	9	5000	1350–10500	141	30–395	2.8
42	9	5160	2100– 7700	76	50–125	1.5
49	7	4450	1400– 8700	86	20–240	1.9
56	10	4970	2350– 9450	58	30–120	1.2
56 (c)	10	3350	1950– 5050	50	15– 90	1.5
63	9	4500	3250– 5800	96	50–165	2.1
70	9	5450	3750– 6600	116	25–175	2.1
90	12	5500	2150– 7950	79	35–145	1.4
90 (c)	5	4060	2700– 7100	35	25– 50	0.9
120	12	4600	1900– 7850	108	50–215	2.3
120 (c)	5	4910	3500– 6500	75	60–105	1.5
180	16	4325	2400– 8900	51	10–150	1.2
240	10	6700	2100–10500	53	25– 75	0.8
240 (c)	5	3100	1900– 5050	25	15– 35	0.8
300	16	5000	2400–10500	67	25–120	1.3
300 (c)	4	4600	2500– 5700	53	30– 75	1.1

Group II. The data for the absolute leukocyte and eosinophil counts of these animals are summarized in table I. A slight leukocytosis was observed in several animals during the second week PI. During the critical stage of the disease, i.e., during the fourth to sixth weeks, animals which had lost the most weight generally showed a slight leukopenia. Note that the leukocytic responses occur before the weight loss.

The absolute number of eosinophils was elevated during the second week and the numbers in some cases remained elevated until the fourth month PI. Division of the absolute eosinophil number by the absolute leukocyte count should produce a quotient approximately equal to the differential eosinophil percentages shown by Group I animals. Treatment of the data in



FIGURES 5–10. Alterations in the differential blood counts of four infected and two normal golden hamsters of Group I. Legend as in previous figures.

this manner was attempted. The highest percentage of eosinophils, however, was 5.2 which was actually only one-fourth of the highest percentage found in stained smears. The highest value obtained from control data was 2.7 percent and all quotients derived from control data were "normal" by differential count standards.

Tissue eosinophilic cells. In routine tissue sections of muscle and skin of infected hamsters the number of tissue eosinophils is also increased. These cells generally have a nucleus with its chromatin masses arranged peripherally so that it resembles the "cart wheel" nucleus of a plasma cell. The cytoplasm contains numerous medium-sized eosinophilic granules. Although the cells are pleomorphic, most appear broadly fusiform or cylindrical. The mean dimensions of the cytoplasm are 15 by 4 microns while the nucleus ranges from 3 to 6 microns in diameter. These cells have been tentatively identified as eosinocytes (Bessis, 1956) although they could almost as readily be termed "Russell body" cells (Kindred, 1932). The cells also resemble the intraepithelial "Schollenleukocyten" of the urinary bladder of the rat infected with *Trichosomoides crassicauda* (Kirkman, 1950).

DISCUSSION

The results above indicate that the hamster, like some other rodents and man, responds to the infection by producing extra circulating and tissue eosinophilic leukocytes. As in the guinea pig (Opie, 1904) the numbers are elevated beginning on the tenth day and reach a maximum during the latter part of the third week PI. By day 10 the larvae have been in the blood for several days (Humes and Akers, 1952). Ritterson (1959) reports that the larval invasion of the musculature begins at the end of the second week. There is no second peak of eosinophilia as has been reported in man by Brown (1898) and Della Vida and Dyke (1941). Like the infected rat and guinea pig (Hamann, 1943; van Someren, 1939) and infected human beings (Huebner, 1911) the intensity of eosinophilia is not correlated with the intensity of the infection.

The percentage of eosinophils in the normal hamster was found to be 0 to 4. Although Byrd (1942) reported no circulating eosinophils in "*Cricetus cricetus*" (*M. auratus?*), this value is in agreement with values reported by Stewart et al (1944), Rose et al (1946), and Trincão et al (1949).

The mean total leukocyte counts of control animals was 4,200 cells per cmm, with a range of 1,550 to 7,500. Crabb and Kelsall (1951) reported a mean leukocyte count of 3,900 with a range of 2,700 to 6,050 cells per cmm of jugular blood. Both of these mean values are low if compared with the data from normal golden hamsters reported by Byrd (1942), Stewart et al (1944), Rose et al (1946) and Sherman and Patt (1956). Sherman and Patt (loc. cit.) and I utilized animals from the same colony but, like Stewart et al (loc. cit.), they obtained blood samples by cardiac puncture and at a different time of day. In other rodents cardiac and peripheral bloods may (Roofe et al, 1950) or may not (Nichols and Miller, 1948) show significant differences in their leukocyte densities.

Since there is no appreciable change in the absolute leukocyte count following infection, the decrease in the percentage of lymphocytes in the differential count indicates that there is an absolute lymphocytopenia and a neutrophilia following infection. Lymphocytopenia has been reported in rat trichinosis by van Someren (1938) whereas Beahm and Downs (1939) found an increase in this type of cell. If one assumes that the lymphocytes do manufacture antibodies (Trowell, 1947) and that the antibodies are released by cellular breakdown (Dougherty and White, 1947), then the observed biphasic lymphocytopenia might be related to the production of the two anti-*Trichinella* antibodies (Wright and Oliver-Gonzales, 1943). A reversal of the lymphocyte : neutrophil ratio has been observed in mice chronically infected with bacteria (Bohme and Bouvier, 1959).

Following infection not only is the number of circulating eosinophilic leukocytes increased but it also appears that the tissue eosinophils are more numerous. Yet most of the tissue eosinophils do not resemble those in the peripheral circulation. Could the tissue eosinophils have originated in situ as Dzury and Cohen (1958) found at sites of injection of antigenic material in rabbits with nitrogen mustard-induced eosinopenia? Or, as is generally thought, are the tissue eosinophils migrants from the peripheral circulation? Let us consider the latter hypothesis. Let us also consider that by some chemotactic and/or selective mechanisms a portion of only the "younger" eosinophils passes out of the vessels into the tissues and metamorphose. The mean age of the circulating population would then be increased. The differential count would show eosinophilia. An absolute count of the eosinophils, however, might not give similar data because the more numerous older leukocytes (including eosinophils) being more sensitive to the hypotonic eosinophil diluting fluid (Lusvarghi et al, 1959) would rupture and not be counted. The disparity of data noted above might reflect the existence of an aging population of eosinophils.

Ordinarily, in human subjects 25 units of ACTH diminishes the eosinophil number about 50 percent. Samter (1955) reports that in several patients with trichinosis counts of 5000 eosinophils per cmm have been reduced to 0 with the same dose. It would seem that the post-infection eosinophil of the peripheral circulation has characteristics different from its normal homologue.

SUMMARY

Trichinosis causes a moderate eosinophilia in the blood of infected hamsters. The eosinophilia reaches a peak from 16 to 19 days post

infection and returns to normal about a week later.

There is a lymphopenia during the second and fifth weeks post infection.

There is no correlation between eosinophilia and weight loss of infected hamsters.

The method used for the determination of absolute eosinophil numbers in the peripheral blood was found not to be as sensitive to changes as the differential count, at least when the technique was applied to blood from infected animals.

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RESEARCH NOTE

GLOBIDIOSIS IN DOMESTIC GOATS.

Alicata (1930, J. Parasit. **16**: 162) stated that the occurrence of *Globidium* in the abomasum of American sheep had not been reported. At that time he recorded the presence of globidia in the abomasum of a number of sheep from Indiana, West Virginia, and Idaho at a percentage of 8.9, 10.8, and 8.3, respectively.

While *Globidium gilruthi* has previously been reported as a parasite of the abomasum, Marsh and Tunnicliff (1941, J. Vet. Res. **2**: 176) first described an acute enteritis in sheep, accompanied by severe diarrhea and associated with a massive infection of the mucosa of the small intestine. Marsh stated that the organism observed apparently corresponds to the species *Globidium gilruthi* in morphology, although that species has hitherto been reported as occurring only in the abomasum

A review of the literature indicates that the occurence of *Globidium gilruthi* in American goats has not been reported. Soliman (1960, J. Parasit. **46**: 29) has reported *Globidium* infections in goats in the Sudan. Other investigators in various parts of the world have reported *Globidium* infections in goats. There follows an account of globidosis in goats from Missouri and Arkansas.

From a herd of 30 goats, composited from an area located within a 100-mile radius of Springfield, Missouri, 10 goats were selected at random and sacrificed. Macroscopic examination of the abomasum of the 10 goats revealed the presence of varied numbers of round, white cysts embedded in the lining of the mucosa. Similar cysts were observed in 4 of the 10 goats. When crushed and stained the cysts were found to contain large numbers of crescent-shaped "spores," rounded at one end and pointed at the other, with a small nucleus near the rounded end. Pinpoint hemorrhages were observed in the abomasum of several of the goats. The protozoan observed apparently is the same species as *Globidium gilruthi* described in sheep by Chatton (1910, Arch. Zool. Exp. et Gen., XIV, 5th Ser., **5** (Notes et Rev. 4): cxiv–cxxiv. Abstracted by Wenyon.). Reichenow and Carini (1937, Arch. Prot. **88**: 374–386), Pellérdy (1960, Acta Veterinaria **10**: 391), and others have suggested that *Globidium* is not a valid genus and the members of this genus should be transferred to *Eimeria*.

Five goats from a herd of 70, composited from an area located within a 35 mile radius of Rogers, Arkansas, were sacrificed. *Globidium* cysts, identical to those described above, were observed in the abomasum of the 5 goats examined. No cysts were noted in the small intestine. —DONALD L. FERGUSON, AND ALICE I. GOLDSBY, *Jensen-Salsbery Laboratories, Kansas City, Mo.*

BIOLOGY OF THE RAT NEMATODE *NIPPOSTRONGYLUS BRASILIENSIS* (TRAVASSOS, 1914). I. SYSTEMATICS, HOSTS AND GEOGRAPHIC DISTRIBUTION*

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Nippostrongylus brasiliensis (Travassos, 1914) has been a popular and valuable experimental animal for nearly three decades. Scores of papers by numerous investigators have dealt with the biology of this nematode, particularly with various aspects of immunity. The present paper is the first of a series, the purpose of which is to summarize and extend the current status of knowledge concerning *N. brasiliensis*.

SYSTEMATICS

Travassos (1914) described briefly and without figures *Heligmosomum braziliense* from the small intestine of *Rattus norvegicus* from Rio de Janeiro, Manguinhos, Brazil. Yokogawa (1920) described *Heligmosomum muris* from the same host species from Baltimore, Maryland. Yokogawa was cognizant of Travassos' paper, but he believed that because of differences in size and shape, *H. muris* was distinct from *H. braziliense*. Later, Travassos (1921) published a more detailed account of *H. braziliense*, including a single figure that incorrectly depicted the male bursa as a symmetrical structure. Travassos did not mention *H. muris* of Yokogawa. He did state, however, that upon examination of the collection of Lutz at the Institute of Bacteriology at Sao Paulo, he encountered material catalogued as *Strongylus spirillum* n. sp. that proved to be *H. braziliense*. Travassos stated further that *Strongylus* sp. of Lutz (1894) also proved to be *H. braziliense*.

Because of the asymmetrical structure of the male bursa and the "bell-shaped ending of the female," Lane (1923) allocated this nematode to a new genus for which he proposed the name *Nippostrongylus*, with *N. muris* as the type. Lane made no reference to *H. braziliense* of Travassos.

Travassos and Darriba (1929), following a study of the literature, claimed that *H. braziliense* and *H. muris* were identical, and that apparent differences between them were due to imperfections in the original description of *H. braziliense* by Travassos (1914). Thus, according to Travassos and Darriba (1929), the correct name of the parasite was *Nippostrongylus brasiliense*. Subquently, Travassos (1937)** changed the spelling of the species name from *brasiliense* to *brasiliensis* and designated *brasiliensis* as the type of the genus *Nippostrongylus*.

Numerous investigators in the United States and elsewhere, particularly the Orient, have continued to use the name *N. muris*. Other workers have accepted *N. brasiliensis* as the correct name of the parasite. In an attempt to reach a definitive settlement of this issue the present author tried to obtain the original material of Travassos and that of Yokogawa for direct comparison. Yokogawa's specimens could not be located and, according to Teixera de Freitas (1956), the type material of Travassos is no longer suitable for study. It has been possible, however, through the very kind assistance of Dr. J. F. Teixera de Freitas of the Institute of Oswaldo Cruz and Dr. David E. Davis, formerly of the Johns Hopkins University, School of Hygiene and Public Health, to obtain topotypic material from *Rattus norvegicus* from Rio de Janeiro, Brazil and Baltimore, Maryland, respectively.

It has been concluded from a study of the topotypic material and an analysis of published

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** Travassos (1937) erroneously used 1921 instead of 1920 as the date of Yokogawa's paper on *H. muris*. The error apparently was due to the fact that a reprint of Yokogawa, S. 1920 A new nematode from the rat. J. Parasit. **7**: 29–33 was published in the Collected Papers, School of Hygiene and Public Health, John Hopkins University (1920–1921) **2**: 29–33.

descriptions and figures that the Brazilian and Maryland specimens belong to the same species. Therefore, as suggested earlier by Travassos and Darriba (1929), *N. muris* (Yokogawa, 1920) is regarded as a synonym of *N. brasiliensis* (Travassos, 1914). A summary of measurements reported by Travassos and by Yokogawa are compared with those from the topotypic material in table I. The Brazilian and Maryland topotypic males also were compared with respect to the structure of the bursa and no significant differences were found between them.

TABLE I. *Comparison of the structure of topotypic specimens of* N. brasiliensis *from Brazil and Maryland with the published descriptions of Travassos and Yokogawa.*

Morphological character	Travassos (1914)	Travassos (1937)	Yokogawa (1920)	Topotypic material*	
				Brazil	Maryland
Cuticular expansion length	—	0.05–0.07	0.06–0.07	0.06–0.07 (0.064)	0.06–0.07 (0.064)
Head diameter	—	—	0.021–0.025	0.019–0.030 (0.0235)	0.019–0.030 (0.0235)
Esophagus length	0.28	0.35–0.48	0.35–0.45	0.29–0.39 (0.345)	0.31–0.39 (0.35)
Excretory pore to base of esophagus		0.06–0.07	0.08–0.14	0.06–0.12 (0.094)	0.06–0.14 (0.094)
MALE					
Body length	2.6–2.8	2.6–4.0	3–4	3.08–4.23 (3.64)	2.68–4.08 (3.36)
Body thickness (maximum)	0.09–0.10	0.08–0.10	0.085–0.10	0.08–0.10 (0.09)	0.08–0.10 (0.09)
Spicules length	0.55–0.56	0.50–0.56	0.56	0.55–0.60 (0.57)	0.45–0.60 (0.54)
Gubernaculum length	—	0.02–0.04	0.06–0.07 0.04–0.05	0.04–0.06 (0.052)	0.04–0.06 (0.051)
FEMALE					
Body length	3.5	2.5–6.0	4–6	3.91–5.28 (4.39)	3.74–5.01 (4.25)
Body thickness (maximum)	0.13	0.09–0.13	0.09–0.12	0.09–0.12 (0.103)	0.09–0.12 (0.104)
Eggs length	0.063	0.054–0.063	0.055–0.062 (0.058)	0.052–0.060 (0.055)	0.052–0.061 (0.055)
Eggs diameter	0.028	0.028–0.034	0.031–0.035 (0.033)	0.028–0.034 (0.031)	0.028–0.034 (0.031)
Vagina length	—	—	0.14–0.16	0.10–0.13 (0.12)	0.11–0.15 (0.13)

* Based on 15 male and 15 female specimens from each locality.

Published descriptions of *N. brasiliensis* include those of Yokogawa (1920), Tubangui (1931), Ko (1938), and Yamaguti (1941) in English, Travassos (1914, 1921, 1937) and Freitas and Lent (1934) in Portuguese, and Skrjabin et al (1954) in Russian. Several of these references are difficult to obtain and in most instances they are either incomplete or partly incorrect. Therefore, it seems desirable to present here a full description of the species. The Portuguese and Russian references were translated and used as part of the sourse material for the following description.

Nippostrongylus brasiliensis
(Travassos, 1914) Lane 1923

Synonyms: Heligmosomum braziliense Travassos, 1914; *Heligmosomum muris* Yokogawa, 1920; *Nippostrongylus muris* (Yokogawa, 1920) Lane, 1923; *Nippostrongylus brasiliense* Travassos and Darriba, 1929; *Strongylus spirillum* Lutz (*in* Travassos, 1921); *Strongylus* sp. Lutz, 1894.

Diagnosis: With the characters of the genus *Nippostrongylus*. Cephalic extremity with cuticular expansion, 0.028 to 0.042 mm in diameter by 0.050 to 0.072 mm in length; cuticle with 14 prominent longitudinal ridges that originate behind cuticular expansion; transverse striations evident in these ridges; head small, 0.019 to 0.030 mm in diameter; mouth simple, buccal cavity small; esophagus conoidiform, sometimes slightly sinuous, 0.280 to 0.480 mm in length; excretory pore situated 0.060 to 0.140 mm from posterior extremity of esophagus; nerve ring about 0.12 to 0.25 mm from anterior end of body; cervical papillae not present.

Male: 2.10 to 4.50 mm in length; maximum thickness near middle of body 0.080 to 0.100 mm; copulatory bursa with small dorsal lobe and two large asymmetrical lateral lobes; right lobe longer than left and strongly incurved; left side: ventro-ventral, latero-ventral, externo-lateral and medio-lateral rays long, thin and gradually tapering; proximal portions of externo-lateral and medio-lateral rays contiguous; postero-lateral ray thick, curved and with conical tip; right side: ventro-ventral ray short and slender; latero-ventral ray moderately long, slender and tapering; externo-lateral and medio-lateral rays thick and contiguous except in distal portion; postero-lateral ray short and slender; externo-dorsal rays short, delicate and arising asymmetrically from common trunk with dorsal ray; dorsal ray bifurcated in distal portion and each branch divided into smaller external branch with simple tip and internal branch with bifurcate or rarely trifurcate tip;

seminal vesicle 0.114 to 0.155 mm in length; spicules yellowish in color, filiform nearly equal, 0.440 to 0.601 mm long and with sickle-shaped extremities; gubernaculum 0.042 to 0.070 mm in length; genital cone prominent.

Female: 2.50 to 6.20 mm long; maximum thickness near middle of body 0.090 to 0.130 mm; posterior portion of body occasionally contracted, appearing swollen and bell-shaped; body terminating in short conical tail, occasionally retracted; single ovary long with small anterior loop; uterus modified anteriorly into seminal receptacle; vagina muscular 0.100 to 0.160 mm long and joined to uterus by well developed ovijector; vulva near anus and both close to posterior end of body; eggs ellipsoidal, thin-shelled 0.052 to 0.063 mm by 0.028 to 0.035 mm.

Habitat and hosts: Jejunum and duodenum of *Rattus norvegicus, R. rattus,* and *Mus musculus.*

Type locality: Rio de Janeiro, Manguinhos, Brazil.

Distribution: Cosmopolitan.

HOSTS AND GEOGRAPHIC DISTRIBUTION

The principal natural host of *N. brasiliensis* is the Norway or brown rat, *Rattus norvegicus,* The known geographic distribution and incidence of infection of *N. brasiliensis* in this host are summarized in table II. The few detailed observations that have been made on worm burdens include those of Harkema (1936) with a range of 1 to 951 and a mean of 247, Filotte (1948) 1 to 290 with a mean of 28, and Schiller and Morgan (1949) 1 to 241. In January 1961, eight infected rats were collected from burrows at the Patuxent Research Center, Laurel, Md. They harbored from 13 to 344 with a mean of 179 *N. brasiliensis* (Huizinga, Solomon, Parker and Haley, unpublished data).

Natural infections of *N. brasiliensis* have been reported also in *Rattus rattus* from Australia (Johnston, 1918b), China (Chen, 1933), Japan (Yamaguti, 1941; Miyazaki, 1946; Tanaka et al, 1951), Formosa (Myers and Kuntz, 1960), Cuba (Perez Vigueras, 1936) and the U.S.A. (Chandler, 1932).

To the writer's knowledge there are only two reports of *N. brasiliensis* occurring in *Mus musculus* in nature; one from Cuba (Perez Vigueras, 1936) and the other in the U.S.A. (Porter, 1935b).

Travassos (1937) and Erickson (1938) listed the deer mouse, *Peromyscus maniculatus,* as a host for *N. brasiliensis.* Both authors apparently based this listing on a note by Porter (1935b) entitled "*Nippostrongylus muris* in the Deer Mouse, *Peromyscus maniculatus.*" Actually Porter's attempt to experimentally infect this host was unsuccessful, but the title of his paper does not indicate this fact. Apparently both Travassos and Erickson knew Porter's paper only by title and, therefore, erroneously inferred that he had obtained positive results.

Recently, Grundmann and Frandsen (1960) claimed to have found *N. brasiliensis* in *Peromyscus maniculatus sonoriensis* and *P. m. rufinus,* and in the long-tailed pocket mouse, *Perognathus formosus incolatus* from Bonneville Basin, Utah. According to Grundmann (personal communication) the parasite was found just three times in some 1200 *P. maniculatus* and once in *Perognathus.* In all four cases only one or two worms were present. Through the generous cooperation of Dr. A. W. Grundmann of the University of Utah the writer was able

TABLE II. *Geographic distribution and incidence of infection of* N. brasiliensis *in Rattus norvegicus.*

Locality	No. examined	No. infected	Percent infected	Author
Australia	400	uncommon	—	Johnston (1918a)
China	69	1	1.4	Wu (1930)
	17	10	58.8	Chen (1933)
	—	—	—	Ko (1938)
	129	4	3.1	Chin (1939)
	30	15	50.0	Lu (1941)
Japan	—	—	—	Kaneko (1938a)
	—	—	—	Miyazaki (1946)
	—	—	39.3	Tanaka et al. (1951)
	—	—	—	Miura et al. (1956)
Philippine Islands	950	551	58.0	Tubangui (1931)
Samoa	—	—	—	Lane (1923)
Egypt	—	—	—	Boulenger (1926)
Spain	—	—	—	Gallego (1959)
England	430	37	8.6	Balfour (1922)
	400	24	6.0	Dudgeon (1922)
Brazil	—	—	—	Travassos (1914)
Panama	400	52	13.0	Calero et al. (1950)
Cuba	—	—	—	Perez Vigueras (1936)
U. S. A.	26	24	92.3	Yokogawa (1920)
	—	—	—	Canavan (1929)
	—	—	—	Chandler (1932)
	2500	421	16.8	Luttermoser (1936)
	55	43	78.2	Harkema (1936)
	40	30	75.0	Cable & Headlee (1937)
	50	2	4.0	Forbes (1942)
	—	—	—	Watt (1943)
	—	—	—	Donaldson & Otto (1946)
	80	50	62.5	Schiller & Morgan (1949)
	14	1	7.1	Hall et al. (1955)
	226	59	26.0	DeGiusti (1957)
	—	—	—	Dickmans (unpublished)
	14	12	85.7	Haley (unpublished)
Canada	150	125	83.0	Firlotte (1948)

to examine a single male and female worm which had been recovered from *P. maniculatus*. These specimens definitely were not *N. brasiliensis*. Specimens from *Perognathus* have not been seen by the writer, but apparently they were the same as those obtained by Grundmann and Frandsen from *P. maniculatus*. It seems very likely, therefore, that the reported findings of *N. brasiliensis* in *Peromyscus* and *Perognathus* were incorrect.

EXPERIMENTAL INFECTIONS

It has been demonstrated experimentally that *N. brasiliensis* can attain sexual maturity in the mouse *Mus musculus* by Porter (1934, 1935a) Kaneko (1938b), Brackett and Bliznick (1947, 1949) Neafie and Haley (unpublished data); the cotton rat, *Sigmodon hispidus* by Lindquist (1949, 1950); the golden hamster, *Cricetus auratus* by Lindquist (1949, 1950), Haley (1954, 1957, 1958a, 1958b); the rabbit *Oryctolagus cuniculus* by Thorson (1953); the chinchilla, *Chinchilla brevicaudata*, and the gerbils, *Meriones unguiculatus* and *Gerbillis pyramidum*, by Haley (unpublished data).

No adult worms were found in the deer mouse, *Peromyscus maniculatus* by Porter (1935b) or the guinea pig, *Cavia porcellus* by Lindquist (1949, 1950) although larvae migrated from the skin to the lungs in both of these hosts. Haley and Lewers (unpublished data) were unsuccessful in attempts to infect the deer mouse, *Peromyscus leucopus*, the vole, *Microtus pennsylvanicus*, and guinea pigs. Recently, patent infections of *N. brasiliensis* were obtained in germfree guinea pigs by Newton et al (1959) and in cortisone-treated guinea pigs by Parker (1961). A detailed consideration of the abnormal host relationships of *N. brasiliensis* will be the subject of a future paper in this series.

SUMMARY

It was concluded from a study of topotypic material and published descriptions that *Nippostrongylus muris* (Yokogawa, 1920) is a synonym of *Nippostrongylus brasiliensis* (Travassos, 1914). This opinion agrees with that expressed by Travassos and Darriba (1929), and others. The chief natural host of the parasite is the Norway rat, *Rattus norvegicus*. *N. brasiliensis* is a common and widely distributed parasite of this host. Natural infections also have been observed in *Rattus rattus* and *Mus musculus*. The results of experimental infections of other animals are discussed.

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RESEARCH NOTE

SUSPECTED ROLE OF PARASITES IN NON-ROOKERY MORTALITY OF FUR SEALS (*CALLORHINUS URSINUS*).

Mortality of fur seal pups on the Pribilof Island rookeries involves a significant proportion of those born each year (Kenyon and Scheffer, 1954, Spec. Sci. Rep., Wildl. No. 12, Fish and Wildl. Ser., pp. 1–77). The hookworm, *Uncinaria lucasi* Stiles, 1901, appears to be of considerable importance in the death of pups on the rookeries and is the only helminth found in them prior to their migration out to sea (Olsen, 1958, Tr. 23d N. Am. Wildl. Conf. pp. 152–175). It is estimated that as many as 400,000 fur seals of all age classes may be lost between mid-August and the following mid-June from natural causes other than uncinariasis (Kenyon and Scheffer, loc. cit.). During this period the fur seal leaves the rookery to feed and is subject to a variety of lethal factors. Relatively little is known in detail about the agencies involved in non-rookery mortality. The present note is concerned with the apparent importance of acute parasitism as one source of "high-seas" mortality.

During early January 1961, three fur seal pups took up residence in the small boat harbor at Valdez, Alaska. They apparently were attracted by schools of herring which had entered the harbor and on which they actively fed. A few days after their first appearance one of them, an 8-months-old male, crawled out on the shore in a very weakened condition and shortly died. Post-mortem examination of the animal revealed that the pup was extremely emaciated, lacking any significant amount of subcutaneous fat, and that it was heavily infected with seven species of helminths. The helminths with numbers and location of each in parentheses are as follows:

TREMATODA: *Pricetrema zalophi* (Price, 1932) (more than 1000 in the small intestine); *Phocitrema fusiforme* Goto and Ozaki, 1930 (one specimen in the small intestine); *Cryptocotyle jejuna* (Nicoll, 1907) (four in the small intestine).

CESTODA: Diphyllobothriid, gen. and sp.? (eight immature specimens in the small intestine).

NEMATODA: *Phocanema decipiens* (Krabbe, 1878) Myers, 1959 (about 200 larvae and four adults in the stomach, 25 larvae in the small intestine and 85 larvae free in the abdominal cavity).

ACANTHOCEPHALA: *Corynosoma strumosum* (Rudolphi, 1802) (six in the small intestine); *Corynosoma* sp. (?) (seven in the small intestine).

Two of the foregoing species probably contributed to the pathology and eventual death of the animal. The larvae of *P. decipiens* which were present in the abdominal cavity had obviously penetrated the wall of the alimentary tract. Although specific lesions were not readily apparent the outer surfaces of the stomach and small intestine were inflamed. The presence of a black, tarry material within the small intestine probably indicates a hemorrhagic condition. Migration within the definitive host of ascaridid larvae of a number of different genera are known to occur, frequently with severe effects on the host. The presumed pathogenic effects of the larva of *P. decipiens* were no doubt aggravated by the presence of large numbers of the minute, heavily spined, heterophyid fluke, *Pricetrema zalophi*. Species of a number of different genera of this family of flukes are known to cause considerable mechanical damage to the epithelial lining of the small intestine. Willey and Stunkard (1942, Tr. Am. Micr. Soc. **61**: 236–253) have described in detail the pathology in dogs of infections of the heterophyid *Cryptocotyle lingua* (Creplin, 1825). The small numbers of the other species of helminths present precludes the possibility that they were involved to any significant extent in the apparent pathology.

To the best of the writer's knowledge, *Pricetrema zalophi* and *Cryptocotyle jejuna* have not been previously reported from the fur seal.—KENNETH A. NEILAND, *Division of Biological Research, State Department of Fish and Game, Juneau, Alaska.*

LABIOSTOMUM COLORADENSIS N. SP. (OXYURIDAE: NEMATODA) FROM THE PIKA, *OCHOTONA PRINCEPS*

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This description is based on eleven complete specimens, three males and eight females, collected by the writer from the large intestine of the pika, *Ochotona princeps,* taken on August 17, 1959, near Gunnison, Colorado.

Description. Body with fine transverse striations and lateral alae. Cephalic inflation of cuticle forming a distinct bulge. Mouth hexagonal, surrounded by six small, conical lips, each supported by four sclerotized bars and armed with a single tooth-like projection. Buccal cavity present, oesophagus club-shaped, terminating in a distinct bulb. Nerve ring at level of termination of cephalic bulge. Valvular apparatus absent; intestine straight and simple.

Male: 3.46 mm long by 0.42 mm wide near middle of the body. Vestibule 0.18 mm deep; oesophagus over-all length 0.78 mm; oesophageal bulb 0.143 mm long by 0.117 mm wide. Length of tail 0.355 mm; caudal alae beginning a short distance anterior to anus and extending to extremity of tail. Tail short, terminating in a sharp point; spicule absent. Seven pairs of caudal papillae; one pair preanal, three pairs adanal, and three pairs postanal with the adanal pairs largest. Longitudinal ventral crests present, beginning at level of cloaca and extending anteriorly for distance of 0.930 mm.

Female: 17.2 mm to 21.6 mm long by 0.67 mm wide slightly posterior to vulva. Diameter of cephalic bulb 0.195 mm, narrow lateral alae extend to posterior to level of anus. Vestibule 0.021 mm deep; oesophagus 1.34 mm long, overall; posterior bulb 0.195 long by 0.182 mm wide. Vulva in anterior part of middle third of body at a distance of 7.80 mm from the anterior end. Tail 1.80 mm long, terminating in a blunt point; eggs asymmetrical, wall double and striated with a plug at one end.

Host: Ochotona princeps.

Habitat: Large intestine.

Locality: Saguache County, Colorado.

Type specimens: U. S. N. M. Helm. Coll. No. 39443.

DISCUSSION

Four species of *Labiostomum* have been described so far. The number and arrangement of the caudal papillae are the most distinct characters by which *L. coloradensis* differs from the other species. *L. akhtari* Inglis, 1959, appears to be similar to *L. coloradensis* in measurements, but the location of the tooth-like, cuticular projections near the lips differ to a considerable extent. These extensions in *L. akhtari* project freely under the midline of each lip directly toward the center of the mouth, whereas in *L. coloradensis* they extend anterolaterally from the adjacent sides of the paired lips. The males of *L. coloradensis* can be differentiated from those of *L. rauschi* Akhtar, 1956, by their smaller size, shorter tail and different arrangement of the ventral crests. The larger size of the body and the ventral crests beginning directly at the anus in *L. coloradensis* separates it from *L. vesicularis* Gvozdev, 1956, which has the ventral crests beginning some distance anterior to the cloaca. The new species differs from the genotype, *L. naimi* Akhtar, 1941, by being larger in size and having fewer caudal papillae. Inglis, 1959, suggested that *Eugenuris* Schulz, 1948, be treated as a synonym of *Labiostomum*. *L. coloradensis* differs from *Eugenuris,* with three species, viz, *E. talkeetnaewus* Akhtar, 1956, *E. schumakovitschi* Shulz, 1948, and *E. pikaeuris* (Akhtar, 1953) Akhtar, 1956, by its inflated cephalic bulb. At this time, the differences are deemed adequate to justify designating the specimens from Colorado as a new species.

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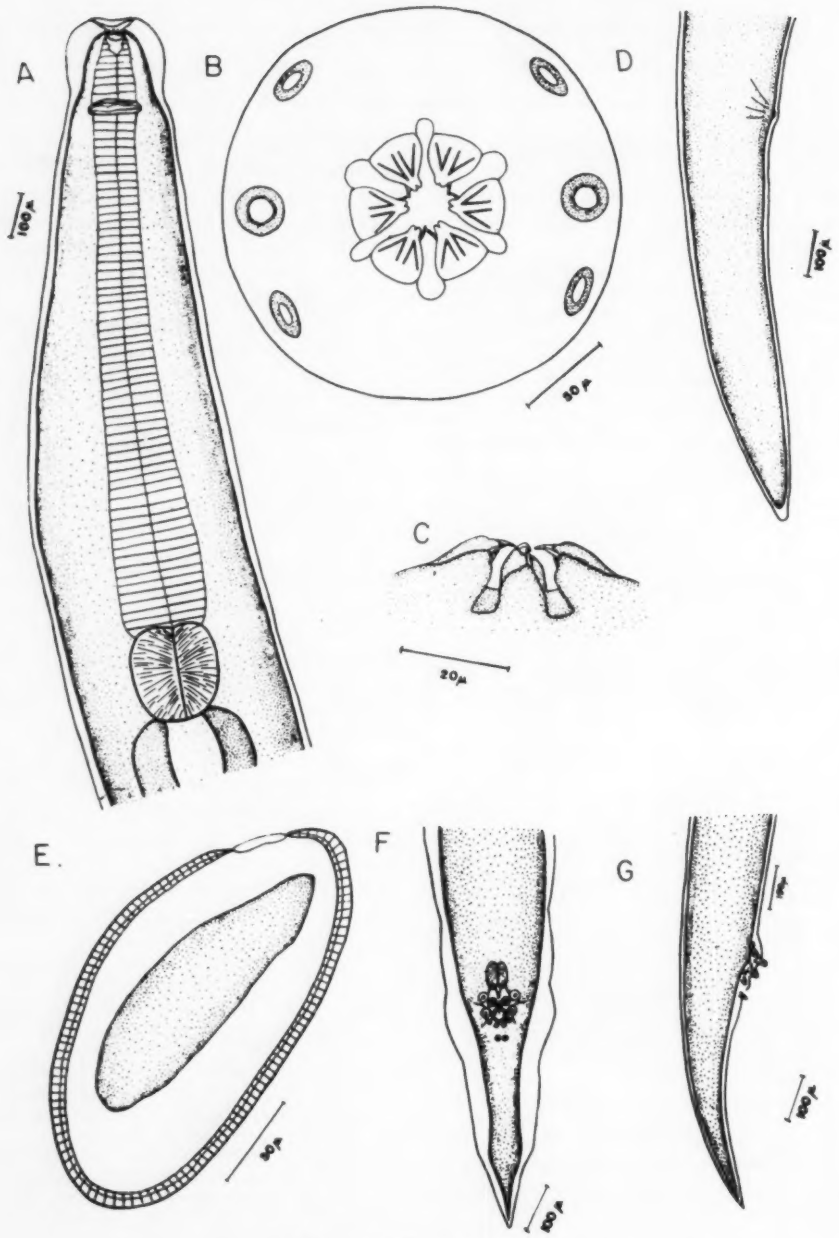
PLATE I



EXPLANATION OF PLATE I

All drawings made with the aid of a camera lucida.

A, anterior end of female *Labiostomum coloradensis;* B, en face view of female; C, lips of female; D, posterior end of female; E, egg; F, posterior end of male, ventral view; G, posterior end of male, lateral view.

THE MOUTH PARTS AND DIGESTIVE TRACT OF ADULT DUNG BEETLES (COLEOPTERA: SCARABAEIDAE), WITH REFERENCE TO THE INGESTION OF HELMINTH EGGS*

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When the eggs of human *Ascaris* and hookworm are ingested by adult dung beetles they are usually destroyed or damaged. The experimental results reported separately (Miller et al, 1961) indicate that helminth eggs in fecal food are destroyed by the action of the mouth parts of *Canthon* and *Phanaeus*, since they rarely appeared at any level of the digestive tract unless they were artificially introduced past the mouth parts by injection. *Pinotus*, on the other hand, swallowed many eggs which survived, although a high percentage were damaged.

The mouth parts and digestive tract of *Canthon laevis* (Drury), *Phanaeus igneus* MacL., and *Pinotus carolinus* (Linn.) were studied comparatively to determine the mechanism of these effects. The three species are essentially alike in the form and function of these organs.

OBSERVATIONS

The Mouth Parts

The mouth parts of dung beetles are of the mandibulate insect type for gathering, masticating, and ingesting solid food. They consist of a labrum, a pair of mandibles, a pair of maxillae, a hypopharynx, and a labium (fig. 2). These parts extend in a horizontal plane on the ventral surface of the head, which is of the prognathous type, and are completely concealed from above by the expanded shovel-like frontoclypeus. The labrum is hinged to the deeply inflected border of the clypeus, mandibles and maxillae are articulated to the ventral margin of the head capsule between the eyes, and the base of the labium forms part of the median ventral wall of the head. The entire labrum and all but the outer basal area of the mandibles are concealed by the underlying maxillae and labium (fig. 1). The hypopharynx, attached to the inner surface of the labium, lies in the oral cavity or cibarium surrounded by the other mouth parts. Excepting the maxillary palpi and the terminal segment of the labial palpi, the exposed parts of the maxillae and labium are densely clothed with hairs which conceivably counteract their tendency to become caked with moist feces and soil and facilitate their cleansing. The oral aperture is surrounded and completely concealed by the bases of the appendages.

While these mouth parts are fundamentally similar to those of leaf-eating scarabaeid beetles (e.g., June beetles) they are markedly modified to adapt them for manipulating the soft moist feces which constitute the usual diet of dung beetles. This adaptation is most conspicuous in the hairy and flexible spatulate blades borne by the mandibles (figs. 2 and 3, inc) and maxillae (fig. 2, Ga, Lc) which gather portions of food. The labrum (fig. 2, Lb) and labium (Lm) are relatively less modified, but their inner surfaces bear numerous spines and hairs that probably serve tractile as well as tactile and gustatory functions. The hypopharynx (Hphy) is a soft, bulky, mobile organ for pushing the food back between the grinding lobes (mol) of the mandibles which lie directly in front of the oral aperture. A median ridge (x) on the hypopharynx, curved to conform with the space between the opposed molar areas, closes the masticating cleft anteriorly when held against the mandibles. (As in most Coleoptera, no salivary glands are evident.)

The mandibles are of special interest since their form and function support the experimental evidence that they are the organs responsible for the destruction of ingested helminth eggs. They are mechanically complex to adapt them for their dual function of gathering and masticating fecal material.

Each mandible (figs. 2 and 3) consists of a

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basal supporting articular portion, a distal incisor lobe (inc) and an inner basal molar lobe (mol). The supporting portion, which is narrowly triangular in side view, bears a dorsal socket (fig. 2, da) and a ventral condyle (figs. 1 and 3, va) which articulate with corresponding structures on the head capsule mesad of the eye. These articular points form a vertical fulcral axis between the insertions of the large adductor muscle (fig. 3, ad) and the smaller abductor muscle (ab) which are attached, respectively, to the inner and outer edges of the base. The mandible, hinged along its laterobasal edge to the head, thus pivots in a horizontal plane. The incisor lobe (inc) is a thin leaflike blade which projects forward horizontally beneath the labrum. It bears a proximal comb of hairs (cmb) and distal fimbriated processes (ifr) along its inner edge, and many small setae on the dorsal and ventral surfaces of the blade. The molar lobe (mol) is massive, projects mesad in a vertical plane, and bears a smooth molar area on the medial surface which opposes that of the other mandible. Varying degrees of sclerotization in the walls of the mandible produce thick, rigid supporting areas and thinner flexible regions. Ventrally a distinctive oval flexible area (fx), consisting of a series of corrugated folds, is present between the incisor and molar lobes.

The two mandibles are symmetrical except for the medial molar area. This is convex on the right mandible (fig. 2, Md′) and concave on the left (Md″), the two molded perfectly to fit closely together. They form an efficient grinding apparatus that functions like a pair of opposed millstones that part to admit grist, come together, and oscillate through a short arc to crush particles between them. These movements, cushioned by the flexible areas of the molar lobes, are imparted by alternate contractions of the mandibular muscles, the abductors swinging the mandibles outward and the adductors inward. The simultaneous motion of the two mandibles thus alternately spreads apart and approximates the incisor lobes to push food medially and at the same time produces the grinding action of the molar lobes upon material that is introduced between them by the anteroposterior movements of the hypopharynx. The special flexible areas (fx) of the mandibles may also enable independent movements of the molar lobes by the adductor muscles while the mandibles are in the closed position.

The molar areas constitute the precise site where food particles and inclusions like helminth eggs are comminuted. The efficiency of titration depends upon the structural characteristics of the mandibles in general and of these areas in particular.

Aside from the shape and direction of curvature of the surface, the left and right molar areas are structurally similar (figs. 4 and 5). The grinding surface is vertically elongate, 4 to 5 times as high as wide. It bears a series of parallel, closely set, transverse cuticular ridges (r), of uniform thickness except for gradual enlargement at the upper end of the molar area. The ridges appear superficially as striations which project posteriorly to form a stiff fringe (mfr) and suggest a file-like surface. Since the ridges extend parallel to the direction in which the molar area moves, it is evident that they cannot function directly as grinding ridges. Closer inspection reveals that the true surface is, in fact, not ridged, but smooth, leathery, and resilient (fig. 6, s).

When the molar area is isolated, cleared, and mounted, with or without pretreatment with potassium hydroxide, high magnification discloses that the ridges are microscopically complex in structure (figs. 6, 7, 8). Viewed from above, they appear cross-striated or barbed, but analytical study of detached fragments of ridges in lateral or transverse aspects reveals that the upper edge of each ridge bears a double series of rectangular lobes. These are contiguous with those of the neighboring ridges and collectively form a smooth microscopic pavement which constitutes the true surface of the molar area (figs. 6, s, and 7). The ridges which carry and underlie these structures have a greater optical density and are thus visible beneath the surface.

The architecture of the ridges is extraordinarily precise and regular. The distal or upper half of the ridge widens and is incised at uniform intervals alternately on each side (figs. 7 and 8, b, c) to produce two parallel rows of prismatic lobes. A ridge fragment viewed from the side thus appears comb-like, with a series of columnar, truncate, and contiguous "teeth" along its upper edge. Viewed from above, these are seen to be the edges of the narrowly rectangular lobes which extend transversely from the midline of the ridge. The lobes on one side of the ridge lie opposite the incisions which demarcate the lobes on the other side. In a ver-

tical cross-section of the ridge the paired lobes form a lamellate scraper-like structure at the summit (fig. 6, t, and 8). Each lobe is vertically striate due to minute parallel grooves on the side which is directed toward the anterior edge of the molar area (fig. 7, e). At the posterior edge of the molar area, only the upper edge of the ridge projects to form a ray of the molar fringe (figs. 4, 5, mfr). It widens and forms a flat blade with a blunt or raggedly toothed tip. On the fringe ray the ridge lobes are replaced by pointed spines which project outward on each side and become sparse distally.

Each of the ridge lobes is a microscopic grinding, rubbing, or triturating instrument, here termed a "tritor" (figs. 7, 8, tri). The tritors are specialized cuticular structures which extend at right angles to the direction in which the molar area moves. Their position and structural characteristics are perfectly fitted to their function. They combine strength and firmness with a degree of flexibility, collectively constitute the entire molar surface, and act as a multitude of stiff scrapers. Food squeezed into a thin film and rubbed between the two closely apposed molar areas is thoroughly triturated by thousands of these minute scrapers acting in unison against those of the opposite mandible. The efficiency of the grinding action is attested to by the destruction of particles as small as helminth eggs and perhaps even protozoan cysts. In this regard, the size of the tritors relative to such eggs and cysts is significant.

Table I compares seven species of dung beetles in regard to body length, dimensions of the molar areas, and size of typical tritors, along with the total number of ridges and tritors on the mandibles. The size of the molar area in each species varies within narrow limits with the size and sex of individual beetles and differs slightly between right and left mandibles. It is here represented by the average lengths of its long and short axes. The tritors show a slight gradation in size according to their position; the dimensions given are averages based on measurements of series on ridge fragments from the central part of the molar area. The number of ridges is, for each species, an approximate average of several pairs of mandibles, and the estimated number of tritors (two rows on each ridge) for both mandibles is derived from the length and number of ridges and the thickness of the tritors.

TABLE I. *Quantitative comparison of the molar structures of seven species of dung beetles.*

Species	Body length average (range) in mm	Molar area (average width and length; mm)	Number molar ridges per mandible (approx.)	Tritors		
				Av. surface dimensions in microns	Av. number per ridge	Total no. on both mandibles
Pinotus carolinus	25.0 (20–30)	0.50×2.4	100	9.0×4.5	200	40,000
Phanaeus igneus	17.5 (13–22)	0.34×1.9	125	7.0×3.5	200	50,000
Canthon laevis	14.2 (10–18.5)	0.32×1.4	100	6.6×2.5	240	48,000
Ateuchus lecontei	6.5 (6–7)	0.13×0.45	55	4.2×2.9	86	9,500
Onthophagus pennsylvanicus	3.5 (2–5)	0.05×0.27	38	3.5×0.9	100	7,600
Aphodius sp.	4.0 (3–5)	0.07×0.13	30	3.0×1.8	70	4,200
Geotrupes blackburnii	16.0 (14–17)	0.28×1.4	100	7.3×4.1	140	28,000

In the species of the subfamily Coprinae, the sizes of the molar areas and of the tritors are directly correlated with the average size of the beetle, all three dimensions decreasing in the series *Pinotus* - *Phanaeus* - *Canthon* - *Ateuchus* (= *Choeridium*) - *Onthophagus*. The large *Pinotus* has fewer and larger tritors than *Phanaeus* and *Canthon*, and its molar area is thus inconspicuously coarser in structure. The small mandibles of *Ateuchus* and *Onthophagus*, with fewer ridges and fewer but smaller tritors, have a much finer structure than those of *Canthon* and *Phanaeus*.

The mandibles of *Aphodius* (Aphodiinae) are similar to the coprine type, except for a shorter and more triangular incisor lobe. The molar area is wider relative to its length and has a smaller area and fewer and thicker tritors than that of *Onthophagus pennsylvanicus* Har., a coprine of similar size. In *Geotrupes blackburnii* (Fabr.) (Geotrupinae) which is comparable in size to *Canthon laevis* and *Phanaeus*

igneus, the mandibles differ markedly in appearance from the *Canthon* type. They are relatively longer, have a massive, rigid, and toothed incisor lobe, a less specialized hirsute comb area, and a less strongly developed molar lobe. There are fewer tritors, and these are slightly larger than those of *Ph. igneus*.

The comparative average sizes of the eggs of *Ascaris lumbricoides* (70 by 47 microns), *Necator americanus* (70 by 38 microns), and *Trichuris trichiura* (52 by 24 microns), the cysts of *Entamoeba histolytica* (12 microns) and *Giardia lamblia* (11 by 7 microns), and the tritors of *Canthon* and *Pinotus* are shown by the diagram in figure 6. In figure 7 outlines of the ascaris egg and amebic cyst are superimposed on the surface view of the molar structures, the solid outlines indicating scale relative to the tritors of *Canthon* and the broken outlines the scale relative to *Pinotus*. The surface area of a tritor in *Pinotus* is about twice that in *Canthon*, so that the longitudinal section of the ascaris egg covers an area equivalent to about 166 tritors in *Canthon* and about 76 in *Pinotus* and the cross section of the amebic cyst an area of about 7 tritors in *Canthon* and 3 in *Pinotus*. These differences in the relative size of the tritors may account, at least in part, for the greater destruction of helminth eggs observed in *Canthon* (and in *Phanaeus*) compared to that observed in *Pinotus*.

The efficiency of the grinding action of the mandibles is also reflected by the size of particles in the paste-like gut contents. These appear coarser in *Pinotus*. In field-collected beetles preserved in alcohol, samples of the midgut contents from four to six specimens of each species showed the following ranges in the size, respectively, of the largest particle and of most of the larger particles (in microns): *Pinotus carolinus*, 6 to 16 and 6 to 12; *Canthon laevis*, 7 to 9 and 5 to 8; *Phanaeus vindex*, 7 to 10 and 5 to 7; *Onthophagus pennsylvanicus*, 3 to 5 and 2 to 4. These particle sizes show positive correlation with the sizes of the tritors listed in Table I.

Although the efficiency of the grinding action must depend upon a combination of mechanical factors, including the precision of occlusion and the amount, nature, and rate of flow of material between the mandibles, it is evidently significantly influenced and limited by the size of the individual tritors that constitute the grinding surface. The mandibles of *Pinotus* are structurally capable of grinding material into particles smaller than *Ascaris* eggs, but they allow many eggs to pass into the digestive tract in a viable condition when they are ingested with feces.

The Digestive Tract

The digestive tract (figs. 9, 10) consists of a short pharynx (Phy) and esophagus (Oe) which constitute the fore-gut; a long, coiled ventriculus (Vent) which is the mid-gut; and a hind-gut which includes an anterior intestine (AInt) and a posterior intestine or rectum (Rect). In *Canthon laevis* the lengths of these divisions in millimeters are: pharynx, 0.5; esophagus, 1.5; ventriculus, 164; anterior intestine, 9; rectum, 1. The mid-gut thus constitutes most of the total length of 176 mm and is about 10 times as long as the body. The proportions are similar in the larger tract of *Pinotus*.

There is no differentiated crop or proventriculus in the fore-gut, and the esophageal valve consists of a simple fold of epithelium into the ventriculus at its junction with the esophagus, as described and illustrated for *Phanaeus* by Beeton (1930).

The ventriculus extends from the prothorax to the base of the abdomen as a straight tube (ThVent), then narrows slightly and becomes a closely coiled mass (AbVent) that occupies most of the abdominal cavity. This compact mass consists of four counterclockwise spiral coils succeeded by five clockwise coils, the smaller central coils lying within the larger anterior and posterior coils, and all held closely together by an abundance of tracheae ensheathed in adipose tissue. Throughout its length the ventriculus bears numerous small papillar crypts (Cpt) that confer a villous appearance to this portion of the digestive tract. These crypts are cylindrical masses of epithelial replacement cells projecting through the lattice-like layer of weakly developed circular and longitudinal muscles which surrounds the epithelial tube.

At the junction of the ventriculus and hindgut is a short muscular pylorie valve (fig. 11, Py) which lies behind the insertions of the four Malpighian tubules. The anterior intestine describes one small clockwise coil which is succeeded by a single large counterclockwise loop that lies dorsal to the ventricular mass and joins the rectum. The anterior intestine and rectum

are provided with conspicuous spiral and circular muscles which surround a layer of longitudinal muscles. The rectum opens to the exterior through the anus which is located at the apex of the abdomen between the pygidium and hypopygium.

The mid-gut and hind-gut are lined by a thin peritrophic membrane (Pmb) which surrounds the intestinal contents and encloses the continuous strand of excrement which the beetle extrudes as it feeds.

The four Malpighian tubules (Mal) lie in a tightly coiled mass within the loop of the anterior intestine and between the adjoining ventricular loops.

There are no grinding structures in the digestive tract. The food material which is finely comminuted by the mandibles is swallowed and passes through the ventriculus as a uniform paste which is subjected to the action of digestive fluids secreted by the epithelium. Absorption of nutrients presumably occurs simultaneously in the ventriculus. Charcoal-laden feces ingested by *Phanaeus vindex* extend throughout the ventriculus within 2 hours. When undisturbed, beetles may continue to feed at the same spot for several hours and extrude coiled strands of excrement which sometimes exceed five centimeters in length.

The digestive tract is adapted by its length and capacty to extract nutriment rapidly from relatively large amounts of the fecal food.

DISCUSSION

Alimentation and Nutrition

The functional modifications apparent in the morphology of the alimentary system of adult dung beetles are readily correlated with the physical and chemical nature of their food. This is typically soft, moist vertebrate fecal material, but some species also eat decaying animal and vegetable matter or fungi (Ritcher, 1958). The beetles are hence fundamentally microphagous (Brues, 1946). The food is swept into the oral cavity by the blade-like distal lobes of the maxillae and mandibles, pushed back by the large mobile hypopharynx, and thoroughly comminuted by the specialized molar areas of the mandibles. Since there are no storage chambers and no indication of microbial symbionts in the alimentary canal, the finely divided food material, enclosed in the peritrophic membrane, presumably moves in a continuous stream through the long, slender, and coiled ventriculus (stomach) and the contained nutrients are digested directly by the beetle. The residue is partly dehydrated in the hind-gut and is excreted as a soft strand surrounded by the peritrophic membrane.

The beetles are voracious feeders and evidently must ingest large amounts of feces to obtain sufficient nourishment. The fecal matter contains undigested food residues, endogenous secretory and excretory products, and bacteria, yeasts, and molds. The chemical composition of human feces, cited by Everett (1946), is approximately 75 percent water, 4 percent fats, 3.5 percent inorganic salts, 2 percent sterols, and 1 percent total nitrogen. Less than 5 percent of the digestible dietary constituents are excreted, and 10 to 25 percent of the fecal mass consists of bacteria. The microbic content of the dung of herbivorous animals is probably even greater. Like coprophagous fly larvae, dung beetles undoubtedly subsist to a considerable degree upon the substance and products of microorganisms that they ingest.

Comparative Morphology

The spatulate maxillary and mandibular lobes and the type of molar areas described are characteristic of the dung-feeding Coprinae and Aphodiinae. They differ markedly from the corresponding structures in plant-feeding scarabaeids of other subfamilies which have stout maxillary and mandibular lobes with rigid teeth for grasping and cutting, and coarse molar ridges for bruising and chopping vegetable tissues (as in the Japanese beetle, illustrated by Swingle, 1930, and Snodgrass, 1950). Examination of the molar areas of several phytophagous species reveals that, in place of the fine horizontal, tritor-bearing ridges found in dung beetles, there are coarse vertical ridges, massive in structure and devoid of tritors. In a pollen-feeder (*Euphoria* sp.) and a sap-feeder (*Ligyrus gibbosus*) neither ridges nor tritors are present on the molar areas which are small non-protuberant plates near the bases of the mandibles. *Geotrupes blackburnii,* which feeds on dung, carrion, and fungi (Howden, 1955), has tritors and spatulate maxillary lobes, but the incisor lobe of the mandible is rigid and bears teeth. (Cf. Hardenberg, 1907.)

The scarabaeid type of coprophagous mouth parts is not found in coprophilic beetles of other families. The mandibles of *Trox* (Trogidae) are hard, short, and massive and have neither molar

lobe nor molar area of the coprine type, while the Histeridae and Staphylinidae have cutting mandibles primarily adapted for a predatory habit. (Coprophagy, however, may be indicated by secondary modifications: in the staphylinid *Baryodma* the cutting edge of the mandible is extended into a thin hairy flange and the maxillary lobes are hairy flaps.)

The digestive tract of dung beetles is relatively much longer than in phytophagous scarabaeids: 8 to 10 times as long as the body in *Phanaeus* and *Canthon*, compared to 1.5 to 3 times in *Phyllophaga*, *Diplotaxis*, and *Popillia* (Becton, 1930; Fletcher, 1930; Jones, 1940; Swingle, 1930). The ventriculus also constitutes a greater portion of the tract in dung beetles (about 88 percent) than in the plant-eaters (50 to 66 percent). The hind gut of dung beetles lacks the specialized area or dilatation of the anterior intestine which is presumably a fermentation chamber in phytophagous species. Fecal food apparently does not require further microbial processing within the beetle for its utilization.

The larvae of Coprinae feed upon buried dung which the adults provide when the eggs are laid. The illustrations of the mouth parts of the larvae of *Pinotus* given by Ritcher (1945) show that they differ markedly from the adult organs. The mandibles and maxillae bear teeth instead of spatulate lobes and are in this way perhaps adapted to cut fecal material that has become consolidated and partly dehydrated. The fine structure of the molar area has not been described, and the efficiency of its grinding action is unknown. The larval mandible of *Geotrupes* (Ritcher, 1947) also differs from the adult mandible. In contrast to the adult, the larva of *Canthon* has a digestive tract only twice as long as the body (Rapp, 1947), although longer than the tract in phytophagous scarabaeid larvae (1.3 to 1.8 times body length: Areekul, 1957). However, like the adult, the larvae has a relatively longer ventriculus than phytophagous larvae and lacks the gastric caeca and (in Rapp's figure) the dilatation of the anterior intestine which are variously developed in phytophagous larvae. In described phytophagous species the larval ventriculus has caeca but no crypts while the adult ventriculus has crypts but no caeca. The physiological and phylogenetic relations reflected by these variations of structure invite further investigation. Detailed comparison of the larval and adult mouth parts of individual species of dung beetles would be of particular interest in the following context.

Ingestion of Parasites

The described structural details of the mandibles and digestive tract elucidate experimental observations, previously reported, that *Canthon* and *Phanaeus* destroy ascaris and hookworm eggs and protozoan cysts present in ingested feces. Their bearing on the oral infection of dung beetles by other parasites must also be considered.

The observed destruction of helminth eggs is directly attributable to the grinding action of the mandibles. The nature of the molar areas explains the ability to destroy microscopic objects in the food, and the absence of other grinding structures in the digestive tract enables eggs to escape mechanical injury when inoculated into the pharynx behind the mandibles.

Similarities in the mandibular mechanism suggest that all species of dung beetles are capable of destroying ingested eggs of ascaris and hookworm. The degree of destruction evidently depends upon the relative efficiency of the mandibles in a given species of beetle, and this seems to be influenced by their size. Although the large molar areas of *Pinotus* have only slightly larger tritors, they allow the passage of many viable eggs, while those of *Canthon*, and *Phanaeus* destroy practically all ingested eggs. No experimental observations have been made on small species like *Onthophagus*, but the small size of the tritors and of particles in the gut would lead one to expect that they, too, could destroy eggs and cysts of the kinds that proved vulnerable in the larger species. The demonstrated efficiency of the medium-sized species indicates that they, at least, do not transport the eggs of human ascaris and hookworm internally.

Dung beetles serve as the intermediate hosts of a number of spirurid nematode and tapeworm parasites of insectivorous and carnivorous vertebrates (Hall, 1929; Morgan and Hawkins, 1949). Spirurid larvae have been reported in species of *Scarabaeus*, *Canthon*, *Phanaeus*, *Pinotus*, *Copris*, *Onthophagus*, *Aphodius*, *Ataenius*, and *Geotrupes*, and the larvae of avian tapeworms in *Choeridium*, *Onthophagus*, *Aphodius*, *Ataenius*, and *Geotrupes* (Seurat, 1916; Ransom and Hall, 1915; Cram, 1931; Alicata, 1935). Spirurid larvae were frequently found

in the body cavity of adult *Canthon, Phanaeus,* and *Pinotus* in the course of the present investigation, but ascaris larvae in embryonated eggs did not survive ingestion by *Canthon.* How, then, do dung beetles become infected with spirurid and cestode larvae despite the ability of the mouth parts to destroy other helminth eggs?

Most of the published records indicate simply that spirurid larvae were found in the body cavity of beetles collected in the field. Seurat (1916) states that they were usually encapsulated third-stage larvae, in one instance 68 *Spirocerca sanguinolenta* and 4,880 *Physocephalus sexalatus* in an individual *Scarabaeus sacer.* However, he also reports finding first-stage larvae of these worms, approaching the first molt, free in the body cavity of adult *S. sacer.* Some of the larvae that we found in other beetles were not encapsulated. Since spirurid eggs contain larvae when deposited in host feces and hatch only in an insect host, these records indicate that dung beetles may ingest them in large numbers and may become infected in the adult stage.

Experimental infection by feeding eggs to adult dung beetles seems to have been reported only for the following: The spururids, *Gongylonema pulchrum* in *Aphodius* spp. (Ransom and Hall, 1915), *Ascarops strongylina* in *Aphodius granarius* and *Physocephalus sexalatus* in *Ataenius cognatus* (Alicata, 1935); and the cestodes, *Raillietina cesticillus* and *Hymenolepis carioca* in *Choeridium histeroides* (Cram and Jones, 1929) and in *Aphodius granarius* (Jones, 1930), *Hymenolepis cantaniana* in *Ataenius cognatus* (Jones and Alicata, 1935), and *Choanotaenia infundibulum* in *Geotrupes sylvaticus, Ataenius cognatus* and *Aphodius* spp. (Horsfall and Jones, 1937, citing Joyeux, 1920, for *Geotrupes*). Except for *Geotrupes,* the beetles infected experimentally are all small species (Aphodiinae and *Choeridium* of the Coprinae).

Seurat (1916) records *Physaloptera abbreviata* third-stage larvae in a larva of *Ateuchus* (= *Scarabaeus*), and Ransom and Hall (1915) found newly hatched larvae of *Gongylonema scutatum* (= *pulchrum*) in larval *Aphodius* spp., but no published record of experimental infection of larval dung beetles has been encountered.

The eggs of spirurids for which dung beetles serve as hosts range from 30 by 11 to 59 by 34 microns, the shells are "thick" (3 microns), and each contains a larva when passed in the vertebrate host's feces which does not hatch until ingested by the arthropod host. The eggs of cestodes known to undergo larval development in the beetles range from 60 by 45 to 88 microns in diameter, have thick envelopes surrounding the smaller onchosphere, and may be enclosed in the adult proglottid when passed by the vertebrate host (Sprehn, 1932). It seems doubtful that the size of these eggs would make them immune to the grinding action of dung beetle mandibles that destroys ascaris and hookworm eggs which are of similar or only slightly larger dimensions. They or the contained larvae must be adapted in some way to withstand mechanical destruction when ingested, perhaps by a resiliency of the walls different from that of the human parasite eggs, or by additional protective coverings around the egg mass. Probably many are, in fact, destroyed during ingestion, while others may be assisted to hatch by the grinding action of the mandibles. Since spirurid eggs may be very abundant in the dung that is eaten in large amounts by beetles, their destruction may prevent excessive and harmful degrees of infection in the insects. The contents of the destroyed eggs would presumably be utilized as food, so that in the encounter between worm and beetle, the beetle would emerge the victor rather than the victim.

The observed difference in *Canthon* and *Pinotus* with respect to the destruction of ingested ascaris and hookworm eggs suggests that species of dung beetles, in the adult stage, may also differ in susceptibility to spirurid and cestode infection in accordance with the grinding efficiency of the mandibles. In the species that have been experimentally infected, mastication may be weaker than in *Canthon* because the mandibles are small (*Aphodius, Ataenius, Choeridium*) or less specialized (*Geotrupes*). The occurrence of spirurids in the large dung beetles could also be the result of ingestion of eggs by these species during their larval stage. Mandibular action may be less efficient in the larva than in the adult.

Further detailed morphological and experimental investigation is needed to resolve the factors underlying the apparent contrast in the fate of different kinds of helminth eggs when they are ingested by dung beetles.

SUMMARY

1. The chewing mouth parts of adult dung beetles are specialized for efficient ingestion and mastication of soft feces. The terminal lobes of the maxillae are spatulate and hairy, the hypopharynx large and mobile. The complex mandible has a flexible, fringed, blade-like incisor lobe and a large molar lobe which bears a smooth, resilient grinding area. This consists of a multitude of precisely arranged triturating structures of a minute size that is directly correlated with the size of the beetle. The asymmetrical molar lobes are closely apposed.

2. The tubular digestive tract consists of a short pharynx and esophagus, a very long, coiled ventriculus lined with a peritrophic membrane, four Malpighian tubules, and a short hind gut. Proventriculus, dilated storage chambers, and salivary glands are absent.

3. Alimentation, nutrition, comparative morphology, and the relation of dung beetles to parasitic helminths are reviewed and appraised. The grinding efficiency of the mandibles, varying with species, may be a significant factor in the susceptibility of beetles to infection by ingested parasites.

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Explanation of Plate

Alimentary Organs of Adult Dung Beetles

Abbreviations used in figures: Ab, abdomen; ab, abductor muscle; AbVent, abdominal ventriculus; ad, adductor muscle; AInt, anterior intestine; An, anus; Ant, antenna; Asc, Ascaris egg (*Ascaris lumbricoides*); Clp, clypeus; cmb, comb of incisor lobe; Cpt, crypt; Ct, cuticular wall of mandible; da, dorsal articular socket of mandible; E, compound eye; Eh, cyst of *Entamoeba histolytica;* fx, flexible area of mandible; Ga, galea (outer terminal lobe of maxilla); Gl, cyst of *Giardia lamblia;* Gu, gula; H, head; Hphy, hypopharynx; ifr, fringe of incisor lobe; inc, incisor lobe; Lb, labrum; Le, lacinia (inner terminal lobe of maxilla); Lf, molar area of left mandible; Lm, labium; LmPlp, labial palpus; Mal, Malpighian tubules; Md′, right mandible; Md″, left mandible; mfr, posterior molar fringe; mol, molar lobe (position of same in figs. 9 and 10); Mx, maxilla; MxPlp, maxillary palpus; Nec, hookworm egg (*Necator americanus*); Oes, esophagus; Pgl, paraglossa (outer terminal lobe of labium); Phy, pharynx; PMb, peritrophic membrane; Py, pylorus; r, molar ridges; Rect, rectum (posterior intestine); Rt, molar area of right mandible; s, molar surface; t, cross section of molar area; Th, thorax; ThVent, thoracic ventriculus; Tr, whipworm egg (*Trichuris trichiura*); tri, tritor; va, vaMd, ventral articular condyle of mandible; Vent, ventriculus; x, median ridge of hypopharynx.

Figure 1. Head of *Canthon laevis,* ventral aspect. 5×

Figure 2. Mouth parts of *Canthon* showing relative positions and cibarial surfaces: ventral or epipharyngeal aspect of labrum (Lb), anterodorsal aspects of right (Md′) and left (Md″) mandibles, and dorsal aspects of maxillae (Mx), hypopharynx (Hphy), and labium (Lm). 16×

Figure 3. Right mandible of *Canthon,* ventral aspect. 16×. Arrow indicates direction of movement.

Figures 4 and 5. Molar areas of right (Rt) and left (Lf) mandibles of *Canthon laevis* (fig. 4) and *Pinotus carolina* (fig. 5), drawn to the scale indicated. 21×

Figure 6. Diagrams showing relative sizes of molar structures of *Canthon laevis* (A) and *Pinotus carolinus* (B) and of helminth eggs and protozoan cysts: r, molar ridges; s, molar surface composed of tritors; t, cross section of ridges and tritors. 90×

Figure 7. Diagram of a portion of the molar surface with superimposed outlines of *Ascaris* egg (Asc) and *Entamoeba histolytica* cyst (Eh) with size relative to *Canthon laevis* indicated by solid outlines and scale line (397×) and size relative to *Pinotus carolinus* indicated by broken lines (257×): a, b, c, successively higher frontal sections of tritor-bearing ridges, with c enlarged to show details of structure. Anterior direction toward right.

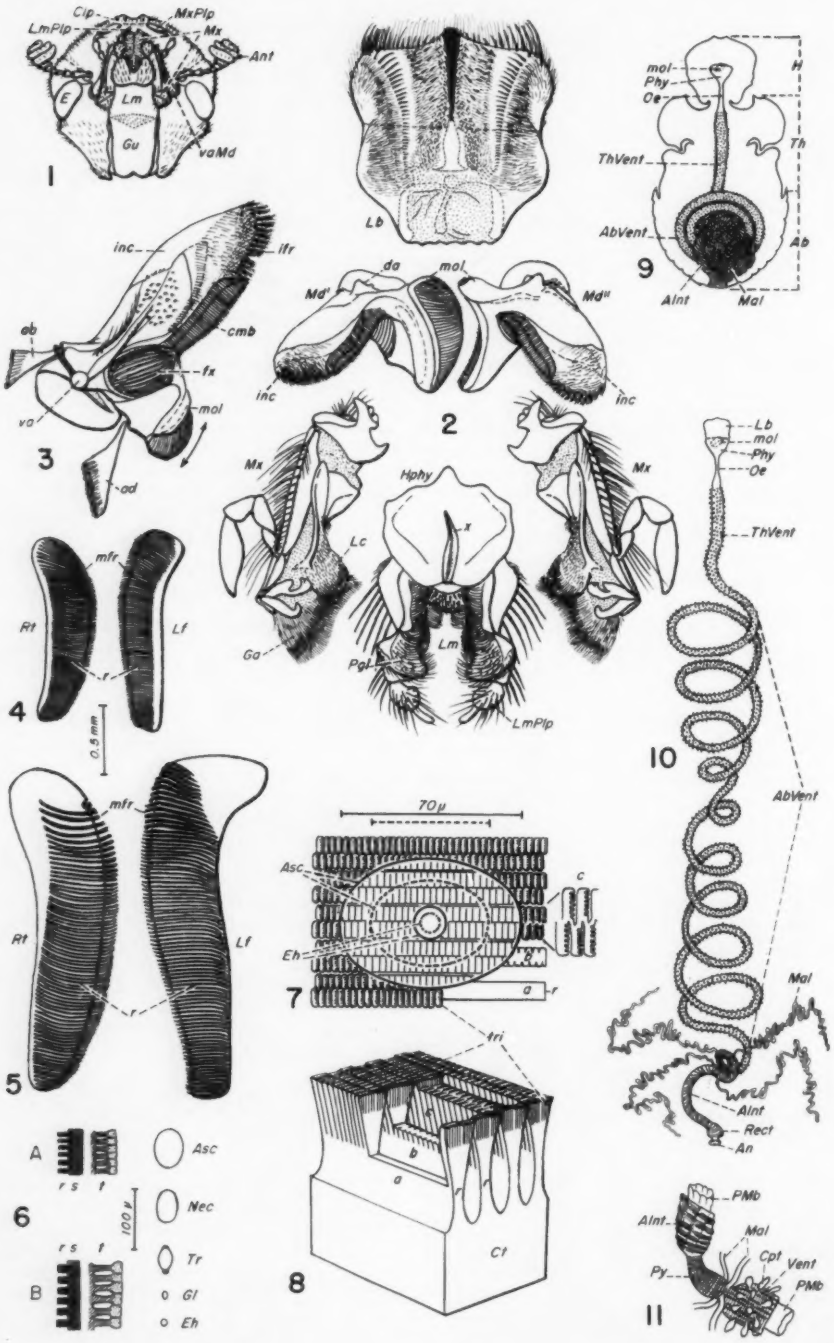
Figure 8. Stereodiagram of the structure of the molar surface, with portions of ridges removed at successively higher levels (a, b, c). (*Canthon,* 397×; *Pinotus,* 257×) Anterior direction toward lower right.

Figure 9. Digestive system of *Canthon laevis,* in situ within body outline. 2.25×

Figure 10. Alimentary tract of *Canthon laevis,* with ventricular coils and Malpighian tubules spread apart. 2.25×

Figure 11. Pyloric region of the alimentary tract of *Canthon laevis,* showing bases of Malpighian tubules and a portion of the peritrophic membrane which lines the tract. 10×

PLATE I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THE FATE OF FOREIGN MATERIALS EXPERIMENTALLY INTRODUCED INTO THE SNAIL *AUSTRALORBIS GLABRATUS**

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The histopathological response of the pulmonate gastropod *Australorbis glabratus* to certain microbial agents (Pan, 1958) implies the existence of mechanisms of tissue defense, but these have been studied mainly in connection with the snails' response to invasion by certain schistosome miracidia (*see* Sudds, 1960, for review). The present report describes the nature of tissue reactions elicited by the introduction of particulate materials into *A. glabratus*.

MATERIALS AND METHODS

A. glabratus of strain PR-1 was maintained on lettuce in aerated distilled water in 10-gallon aquaria, or 3-liter Pyrex battery jars, under continuous light, at a temperature of 76 ± 2 F (Chernin et al, 1957).

The following particles were injected: Yeast (dried and cultured), bacteria, erythrocytes, carmine, pollen, and polystyrene spheres. Except as noted, water redistilled in glass was used for washing by centrifugation and to resuspend particles prior to injection. The reactions of snails to implanted homologous and heterologous tissues were also studied.

Preparation of Materials for Injection or Implantation

Yeast. *Saccharomyces cerevisiae* was derived from a single package of dried baker's yeast (Fleischmann's). The yeast was washed once and (a) left untreated, (b) resuspended in nutrient broth and autoclaved (15 lbs. pressure for 15 min), or (c) resuspended in distilled water or nutrient broth and held in a water bath at 100 C for 15 minutes (hereafter referred to as "heat-killed" yeast). In each instance these preparations were washed 3 times and the final volumes adjusted to contain approximately 0.2 ml of packed cells in 10 ml of distilled water.

In addition to the foregoing, a laboratory culture of the yeast was derived from the commercial product by culturing at 37 C in Sabouraud's broth or agar containing penicillin (40 units per ml) and streptomycin (40 micrograms per ml). This was subcultured thereafter on Sabouraud's agar, with or without antibiotics. For experimental use, it was cultured for 48 hours in Sabouraud's broth, harvested by centrifugation, and treated in each of the three ways indicated above.

Final suspensions of the dried or the laboratory-cultured yeast contained cells which were characteristically Gram-positive and uniform in shape and size (2.5 by 5.0 microns).

Bacteria. An unidentified Gram-positive coccus (1.5 microns in diameter, occurred in pairs or short chains) was isolated from the dried commercial yeast preparation by culturing in Sabouraud's broth at 37 C. Subcultures of bacteria harvested by centrifugation were treated in each of the three ways outlined above for yeast, and the final volumes adjusted to contain a 2 percent (approximately) suspension of cells by volume.

Erythrocytes. Freshly drawn chicken heart blood was allowed to clot and placed in 10 percent formalin in normal saline at 5 C for about 16 hours. (Fixation was necessary to prevent hemolysis upon injection of erythrocytes into snail blood.) Clots were then fragmented mechanically and the freed erythrocytes (average size, 3.7 by 8.5 microns) were washed 4 times in saline. The final suspension was adjusted to contain 0.5 ml of packed erythrocytes in 4 ml of distilled water.

Carmine. Grübler carmine was prepared as a 1 percent suspension (by weight) in distilled water, and washed once before use. Particles measured 1.0 to 2.5 microns.

Willow pollen. *Salix nigra* pollen was washed twice and resuspended in distilled water in a final concentration of 2 percent by volume. Particles averaged 19 by 24 microns.

Polystyrene spheres. Dow Chemical Company "8 to 16 microns" polystyrene latex spheres (average diameter, 15 to 18 microns) were washed in water 12 times and suspended in a final concentration of 5 percent by volume.

Tissue implants. Tissues for implantation were removed from *A. glabratus* and from another pulmonate, *Planorbarius corneus*, which had been colonized in 1959 from snails collected in Wellesley, Mass. (Chernin, 1959, pers. com.). Both strains of snail were maintained as described above. Freshly dissected foot tissues from these snails were treated in one of the following ways: (a) Tissues

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from *A. glabratus* were fixed in 10 percent neutral formalin for 4 days, then washed in running tap water for 1 day and in six changes of distilled water for another day. The tissues were then divided into several fragments of approximately 4 to 8 cu mm in volume; (b) unfixed tissues from *A. glabratus* and *P. corneus* were washed for 4 hours in several changes of distilled water and divided into fragments as above.

EXPERIMENTAL PROCEDURES

Groups of 15 to 20 snails (average diameter, 10 to 15 mm) were narcotized by placing them in 150 to 200 ml of freshly prepared 1 percent urethan. After 3 to 4 hours at 25 C, narcotized snails were secured individually in a dish and covered with distilled water. A small glass hook was inserted into the male genital orifice and sufficient tension applied to expose the neck of the snail and thus allow access to the region of the cephalopedal sinus. The details of this technique have been described by Michelson (1958).

Each inoculum (0.05 to 0.10 ml) was injected slowly (10 to 20 seconds) into the cephalopedal sinus by means of a 30-gauge needle and a 0.25-ml syringe. Varying amounts of the injected material escaped upon withdrawal of the needle, but immediate release of tension on the neck of the snail permitted the snail to withdraw into its shell and thus close the wound. Snails which retracted slowly or only partially usually died subsequently. Control animals received injections of similar volumes of distilled water and were then treated in the same manner as experimental snails.

For purposes of tissue implantation, a longitudinal incision 5 mm long was made with a scalpel in the lateral surface of the neck over the cephalopedal sinus. The tissue fragment, held by fine forceps, was inserted into the sinus and the edges of the incision were allowed to close; snails were then returned to aquaria for maintenance. Controls included normal and sham-operated *A. glabratus* and normal *P. corneus*.

Preliminary experiments indicated that nearly all narcotized snails, both experimental and control, moved and fed within 1 to 2 hours after their return to aerated aquaria. Snails which did not recover in 4 to 6 hours were discarded.

In experiments involving injection of particles, pairs of snails were usually sacrificed at the following intervals after injection: 10 minutes; 2, 4, 8, 16, and 24 hours; and 2, 4, 8, 12, 16, and 20 days. In implantation experiments, the first and second pairs of snails were killed at 2 and 24 hours, respectively. All snails were sacrificed by placing the intact animals in Zenker's fixative for 15 minutes; the shell was then removed and the body returned to the fixative for an additional 18 to 24 hours. The tissues were then washed, dehydrated in ethanol, cleared in xylol, embedded in paraffin and sectioned serially at 10 microns. Eight alternate strips, each representing 6 to 8 sagittal sections, were stained from each snail. Gram's tissue stain was used when yeast or bacteria were to be sought in the tissues, while hemalum and eosin were used to stain sections containing other materials. Hemalum-azure II-eosin and Giemsa stains were also employed occasionally.

Because of unavoidable postinjection leakage of blood and inoculum, it was not possible to quantify the number of particles injected. However, this shortcoming had no apparent effect upon the results outlined below since experimental findings were consistent both within any one experiment and between comparable experiments.

RESULTS

Large numbers of particles were seen in the sinuses of such highly vascularized tissues as the rectal ridge, the mantle-kidney, and the mantle collar. This suggests that all particulate material injected into the cephalopedal sinus passed into the adjacent confluent sinuses where most of it was retained. Fewer particles were present in the sinuses of such dense tissues as the foot, or in connective tissues between organs. In most cases, the particles in sinuses were in close proximity to amoebocytic cells.

Yeast. Phagocytosis of yeast cells trapped in connective tissues began within 10 minutes of injection in all experiments and was virtually complete (est., 95 percent) within 8 to 16 hours. Most amoebocytes contained 1 to 3 yeast cells, although 6 to 8 cells per amoebocyte were commonly seen, and up to 20 yeast cells were counted in occasional hypertrophied amoebocytes.

Beyond phagocytosis, the further defense reaction of the snail depended on the prior treatment of the injected cells and entailed one or both of the following processes: (a) Migration of particle-laden phagocytes to and through certain epithelial layers to the external aquatic environment; and (b) intracellular breakdown of particles as evidenced by cellular fragmentation and failure to take up stain.

The responses of *A. glabratus* to dried or cultured yeast cells (comparably pretreated) were basically similar. Approximately equal numbers of untreated yeast cells were removed by (a) constant migration of yeast-laden amoebocytes through epithelium to the external environment during a period not in excess of 20 days, and (b) intracellular degradation, a process which was most evident at 4 days. By contrast, most "heat killed" yeast cells were removed by migrating amoebocytes during the first 2 days after injection (fig. 1), while the few yeast cells remaining in the tissues at 4 days were degraded intracellularly. Nearly all

autoclaved yeast cells were degraded intracellularly within 4 days postinjection and only rarely were they seen in epithelial layers. Untreated yeast cells persisted in the tissues longer than either "heat killed" or autoclaved yeast cells.

Bacteria. Injected bacteria were eliminated from the tissues in much the same way as yeast treated comparably. Some untreated cocci were seen in epithelial layers, usually at 8 to 24 hours, and occasionally through 16 days. Intracellular degradation was a constant finding from 4 hours through 22 days. Many "heat killed" cocci were seen in epithelia at 8 to 48 hours, but intracellular breakdown was seen only at 12 days when few cocci remained in the tissues. Autoclaved bacteria were rarely seen in epithelial layers; intracellular degradation was evident from 2 to 23 days, although some bacteria were still present in the tissues after 36 days.

Chicken red blood cells. Formalin-fixed erythrocytes were phagocytized rapidly and were degraded completely within 96 hours after injection. Snails sacrificed 10 minutes after injection contained large numbers of red blood cells with dark blue, evenly-stained, well-defined nuclei surrounded by clear pink cytoplasm and a well-defined cell membrane. At 2 hours the nuclei of some of the intracellular erythrocytes were pale blue, slightly uneven, and surrounded by poorly stained cytoplasm and distorted cell membranes. Signs of advanced degeneration of erthyrocytes (pale, blue-green nuclei; pale yellow cytoplasm; no demonstrable cell membrane) were noted at 24 to 72 hours. Tissue sections of snails sacrificed at 4 to 28 days were negative for erythocytes. No evidence was obtained to indicate that erythrocyte-laden leukocytes migrated through epithelium.

Carmine. Clumps of carmine were present in amoebocytes near the injection site for 30 days postinjection. No carmine was seen in epithelium at any time.

Pollen and polystyrene. A. glabratus reacted to injected pollen or polystyrene spheres in a characteristic and similar fashion. Although the size of these particles precluded phagocytosis, amoebocytes were found stretched around the periphery of most particles within 16 to 24 hours of injection (fig. 2). Beginning nodule formation was evident by 24 hours as fibroblasts migrated from adjacent connective tissue (fig. 3). These processes resulted in a well-defined lesion, circular in section, consisting of a central mass of densely packed particles and amoebocytes surrounded by one or more layers of fibroblasts (figs. 2 and 4).

By 4 days most pollen grains were within such nodules (fig. 2) or, as individual particles, had been surrounded by a layer of amoebocytes. Such lesions persisted for at least 42 days without any evidence that the contained pollen had undergone change. Similar processes culminated in the isolation of polystyrene spheres, but formation of discrete nodules walling off the majority of these particles took up to 22 days, probably because of the larger number of spheres involved.

Tissue implants. Formalin-fixed *A. glabratus* tissue was encapsulated, within 24 hours after implantation, by fibroblasts migrating from nearby connective tissue. The capsules became increasingly thickened, but remained otherwise unchanged, for 30 days (fig. 6). On the other hand, implantation of fresh *A. glabratus* tissue elicited no fibroblast response (fig. 5), and only a transient amoebocytic infiltration, the latter probably in response to damaged and necrotic cells. At 20 days the implant was pale-staining, but it was intact and its epithelium had fused with that of the host at the edge of the incision (fig. 7).

Fresh *P. corneus* tissue was also encapsulated by fibroblasts within 24 hours after implantation. However, progressive atrophy of cells of the implant occurred and, after 26 days, only a poorly organized fibrotic mass remained. The implant was surrounded only by scattered groups of fibroblasts, and the implant itself became invaded by fibroblasts and muscle cells which had migrated from adjacent host connective tissues (fig. 8).

DISCUSSION

Injection of particulate material into the cephalopedal sinus of *A. glabratus* largely limits its distribution to the blood spaces of anterior connective tissues. The ensuing cellular reaction is modified by the size and the nature of the particle. Particles small enough to be phagocytized are quickly engulfed and may be (a) transported through epithelial surfaces to the outside, (b) degraded intracellularly, or (c) retained within tissue amoebocytes.

The migration of particle-laden amoebocytes to the exterior presumably accounts for the

presence of particles in epithelial layers. Yeast cells were most frequently seen in the pigmented outer epithelium of the mantle and mantle collar, in the inner surface of the mantle above the mantle cavity, and in the epithelium of the rectal ridge bordering the mantle cavity. Yeast cells were infrequently seen in the epithelium of the digestive gland of the rectum, or in the pigmented epithelium subjacent to the columellar muscle. The frequency with which yeast cells were seen in these epithelial areas was related to the abundance of injected yeast cells in blood spaces adjacent to the respective tissues.

The fate of a phagocytized particle is appreciably determined by its nature and previous treatment. At one extreme, for example, an inert material such as carmine remained unaltered within tissue amoebocytes. More labile particles, such as untreated or "heat-killed" yeast, and bacteria, were removed by various combinations of migration and degradation. At the other extreme, autoclaved yeast and formalin-fixed erythrocytes were subject only to intracellular degradation. Particles too large to be engulfed by amoebocytes, and therefore not subject to elimination either by intracellular degradation or by migration, elicited an initial amoebocytic response which was reenforced by fibroblastic encapsulation and nodule formation.

The biological importance of these complex, rapid and apparently efficient defense mechanisms remains unknown, although it might be assumed that such a highly developed response serves some important function under natural conditions. Two additional points are noteworthy. First, when bacteria originally isolated from naturally infected snails (*Bacillus pinottii*) (Cruz and Dias, 1953) were injected into *A. glabratus*, the organisms could not be found in tissue sections after 1 hour, nor could they be cultured from snail tissues after 48 hours (Tripp, 1961). The defense mechanism responsible for the elimination of this organism has not been elucidated nor was there any associated pathology. On the other hand, in experimental infections of *A. glabratus* with *Mycobacterium* sp. (Michelson, 1960), also isolated from snails, host tissue reactions involve elements of the defense processes described above. In this case, the bacteria were phagocytized, multiplied intracellularly, and masses of infected cells accumulated to form the nucleus of each fibroblastic nodule. It appears that since amoebocytes are unable to eliminate these bacilli either by migration or by degradation, the mechanism of nodule formation is called into play.

It is of some interest to note the basically similar responses to injected particles on the part of *A. glabratus* (a fresh water gastropod) and of a marine pelecypod (*Crassostrea virginica*). Although these studies were done at different times and with different materials, viable bacteria were not demonstrable in the tissues of either mollusk more than 48 hours after injection (Tripp, 1960, 1961). While injected avian erythrocytes were phagocytized immediately in both mollusks (Tripp, 1958, and present report), intra-amoebocytic lysis took place much sooner in the snail injected with fixed red cells (2 to 4 days) than in the oyster injected with fresh red cells (12 to 16 days). Untreated yeast cells were removed by 12 days from oyster tissues by migrating amoebocytes (Tripp, 1960), but in the snail a similar migratory process encompassed at least 20 days and was supplemented by intracellular degradation. It thus appears that these two mollusks possess similar defense mechanisms and that in both instances amoebocytes play the primary role in ridding the tissues of injected particles. The relative importance and rapidity of the subsequent migratory and digestive processes (when these occur) differ somewhat in the two animals.

Tolerance by the snail of implanted homologous tissue was striking in that the initial minimal amoebocytic response was followed by integration of the intact implant with host tissue. On the other hand, heterologous tissue from *P. corneus* was encapsulated in *A. glabratus*, subsequently deteriorated, and became infiltrated by host fibroblasts and muscle fibers. Thus, *A. glabratus* appears to discriminate between homologous and heterologous tissue.

Both the injection and the implantation procedures reported above involved varying degrees of trauma to the neck and head-foot tissue of the snail, and were frequently accompanied by extensive hemorrhage. However, at least 90 percent of snails subjected to these operations recovered, appeared normal within a few hours, and continued so for as long as 3 months. Furthermore, injections of as much as 0.10 ml of water, an amount which probably

represents a rather large proportion of the snail's total blood volume, apparently had no adverse effects on subsequent survival rates. The capacity of *A. glabratus* to tolerate such gross insults commends this snail as an experimental subject for histopathologic studies of the kind reported herein.

CONCLUSIONS

1. Foreign materials were introduced by injection or implantation into the cephalopedal sinus of the pulmonate snail, *Australorbis glabratus*, and the variety of host responses elicited were studied in tissue sections.

2. Particles small enough to be phagocytized were removed from the tissues by the migration of host amoebocytes through epithelial layers to the exterior, by intracellular degradation, or by retention within tissue amoebocytes. Particles too large to be phagocytized (pollen and polystyrene spheres) were walled off in capsules of fibroblasts.

3. The response of *A. glabratus* to implanted tissues was investigated. Differences in response are described which seem to depend on whether the tissue was fresh or fixed, and whether it was homologous or not. Fresh homologous tissue elicited no response; fixed homologous tissue was encapsulated, and fresh heterologous tissue (from *Planorbarius corneus*) was destroyed in *A. glabratus*.

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EXPLANATION OF PLATES

PLATE I

FIGURE 1. Yeast cells ("heat killed") in mantle collar epithelium (a) of *A. glabratus* 4 hours after injection. Numerous yeast-laden amoebocytes are visible in subjacent blood sinus. Gram stain. 500 ×.

FIGURE 2. Well-defined nodule in connective tissue of mantle collar containing several pollen grains injected 4 days earlier. Note prominent peripheral fibroblasts. Hemalum and eosin. 500 ×.

FIGURE 3. Cluster of polystyrene spheres and amoebocytes of *A. glabratus* in cephalopedal sinus 2 days postinjection. Hemalum and eosin. 250 ×.

FIGURE 4. Large lesion in foot of *A. glabratus*, around polystyrene spheres injected 12 days earlier. Hemalum and eosin. 200 ×.

PLATE II

FIGURE 5. Section through cephalopedal sinus of *A. glabratus* in which a piece of unfixed *A. glabratus* foot tissue (a) had been implanted 2 hours earlier. Note the lack of host-cell response and normal architecture of implant, typical for this and for later samples. Hemalum-azure II-eosin. 36 ×.

FIGURE 6. Section through cephalopedal sinus of *A. glabratus* containing formalin-fixed *A. glabratus* foot tissue (a) implanted 20 days earlier. Note complete encapsulation of implant. Hemalum and eosin. 36 ×.

FIGURE 7. Unfixed *A. glabratus* foot tissue implant (a) "grafted" to host tissue (b) at edges of incision. Twenty days after implantation. Note fusion of implant and host epithelium. Hemalum-azure II-eosin. 100 ×.

FIGURE 8. Unfixed *P. corneus* foot tissue implant (a) infiltrated by host (*A. glabratus*) fibroblasts and muscle fibers (b). Twenty days after implantation. Hemalum and eosin. 100 ×.

PLATE I

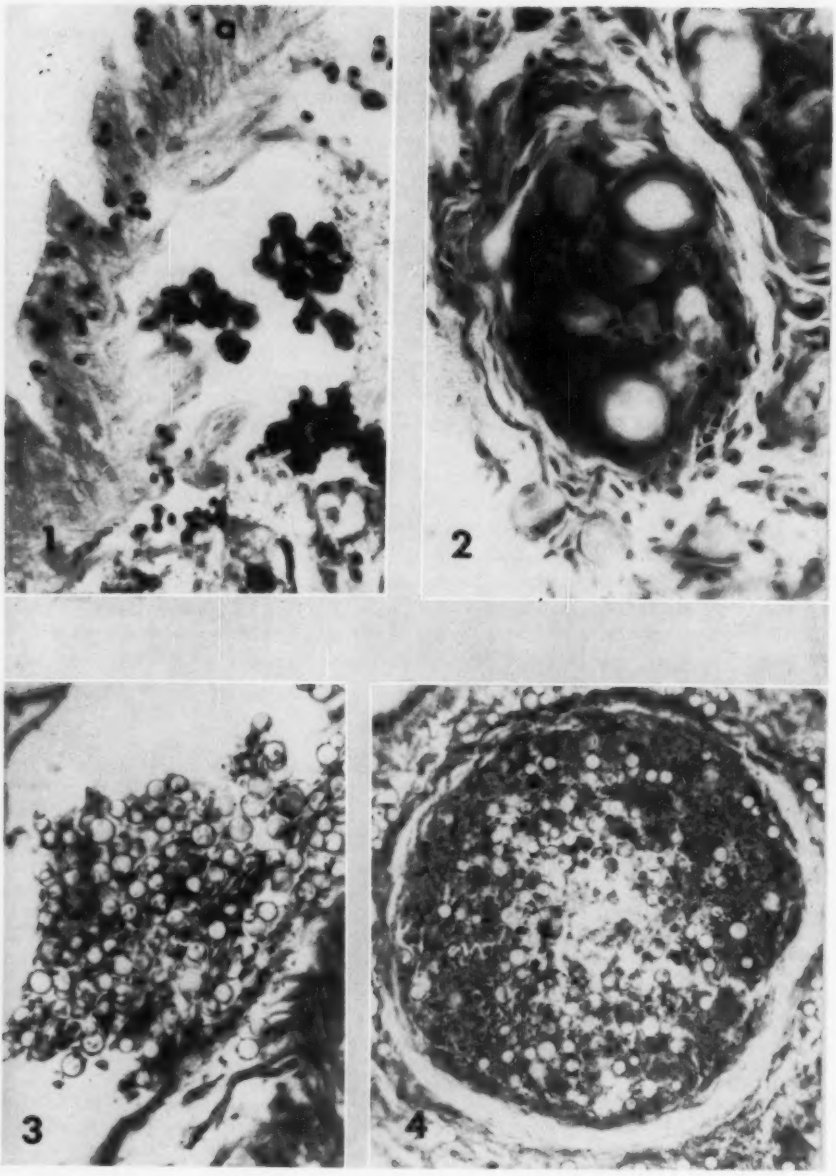
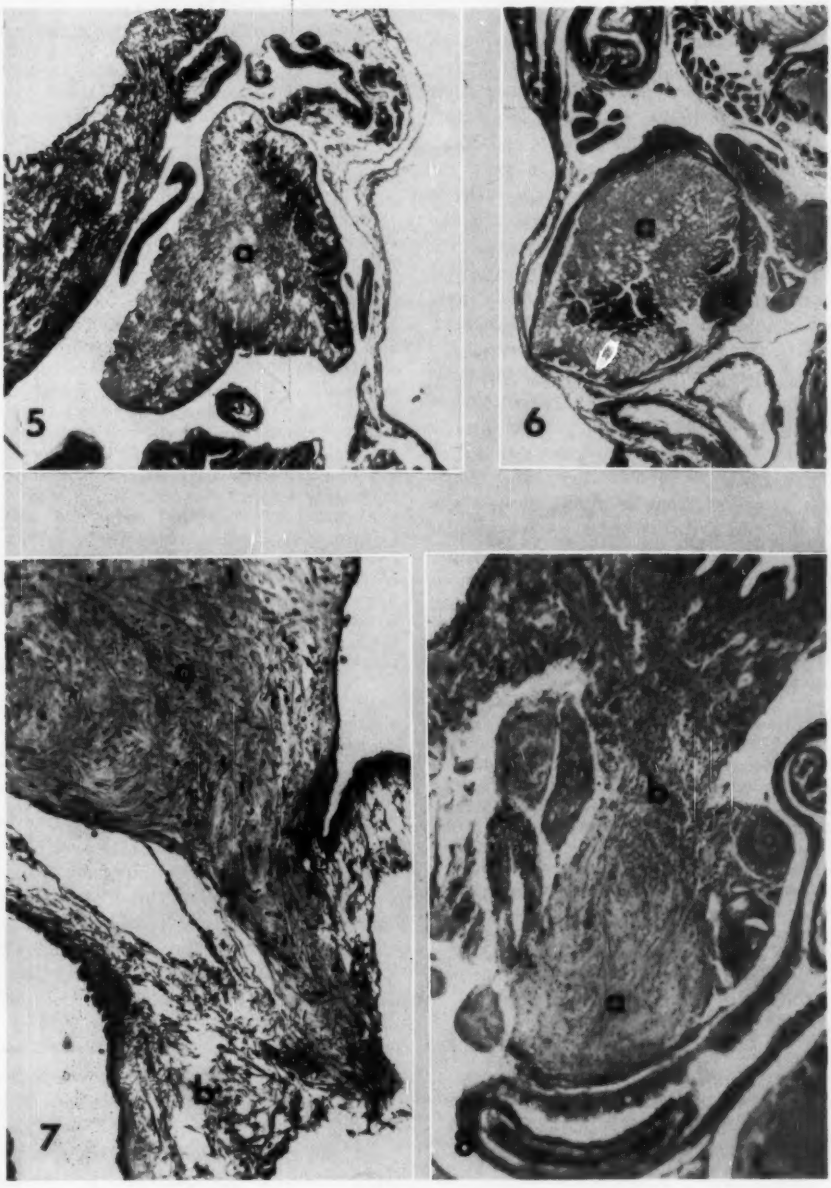


PLATE II



SUSCEPTIBILITY OF WILD MAMMALS TO INFECTION BY *SCHISTOSOMA MANSONI*

JOHN I. BRUCE*, LEONARD M. LLEWELLYN** AND ELVIO H. SADUN*

In the past it was believed that man was the only epidemiologically important mammalian host of *Schistosoma mansoni*. Recent studies, however, have shown that a few other hosts may play a role in the dissemination of this parasite (Barbosa et al 1958; Kuntz and Malakatis, 1955; Price, 1953; Martins, 1958).

The present investigation was undertaken to determine if wild mammals trapped in the Washington, D. C.-Baltimore, Maryland, area were susceptible to *S. mansoni*. It was regarded as unlikely that any of these animals were naturally infected because no recognized snail host of *S. mansoni* is present in this area. The degree of susceptibility of the various mammals tested was determined by the percentage of parasites developing in the host; the growth and structural development of the worms; the ability of worms to produce eggs; the viability of eggs recovered from the feces, intestine and liver; the infectivity of miracidia for suitable snails; the sex ratio and location of the worms in the host; the length of the prepatent period; and the pathological manifestations of infection.

MINERALS AND METHODS

Mammals of the following species were examined for the presence of natural infection, and after having been found uninfected were exposed to counted numbers of cercariae of *S. mansoni*: *Orders*: RODENTIA, *Marmota monax*, woodchuck; *Myocaster coypus*, nutria; *Tamias striatus*, eastern chipmunk; *Sciurus carolinensis*, grey squirrel; *Peromyscus leucopus*, white-footed mouse; *Microtus pennsylvanicus*, meadow vole; *Ondatra zibethicus*, muskrat; *Rattus norvegicus*, house rat; *Zapus hudsonius*, meadow jumping mouse. CARNIVORA, *Procyon lotor*, raccoon; *Mephitis mephitis*, striped skunk; *Vulpes fulva*, red fox LAGOMORPHA: *Sylvilagus floridanus*, eastern cottontail. MARSUPIALIA: *Didelphis marsupialis*, the opossum.

Cercariae of the Puerto Rican strain of *S. mansoni* were obtained from pools of no less than 40 infected snails (*Australorbis glabratus*). The mammals were infected by placing the cercariae in contact with the shaved abdominal area for 45 minutes. The smaller rodents were exposed to 200 cercariae each. In the larger animals the exposure was graded according to size, with a maximum of 1000 cercariae in the largest. All animals of the same species were exposed to the same number of cercariae. Albino laboratory mice were used as controls with each infection and were exposed to 200 cercariae each.

All mammals, except the nutria, were trapped in the Washington, D. C.-Baltimore, Maryland, area. The nutria were obtained from Florida. The age of the animals was not constant, although when feasible, hosts of medium size were selected. The animals were necropsied after 8 to 12 weeks, except that some were held longer to determine whether more time was necessary to permit egg depositions in the intestinal tract. The animals were perfused by the "Perf-O-Suction" technique described by Radke et al (1961). After perfusion, the liver and intestine from each animal was removed and examined microscopically in pressed preparations for the presence of eggs, pigmentation, and other evidence of infection.

Bi-weekly fecal examinations were started in the seventh week after exposure. Each fecal sample was studied by both direct smear and AMS III (Hunter et al, 1948) concentration techniques. The viability of eggs from the feces, intestine, and liver of the animals was determined by the hatching technique. The infectivity of miracidia was determined by exposing them to susceptible *A. glabratus* snails in a concentration of approximately 10 miracidia per snail.

Intact parasites were measured within 1 hour after their removal from the host. The measurement was performed with the aid of a dissecting microscope and a ruler placed beneath the slide.

RESULTS

None of the mammals studied prior to experimental infection was found to pass *S. mansoni* eggs within a period of approximately 2 months after trapping. One-fourth of the animals trapped were sacrificed approximately 2 months after being trapped, the others were exposed to *S. mansoni* cercariae. At necropsy, no schistosomes were found in any of the animals which had not been experimentally exposed to cercariae.

Sixty-eight wild mammals were exposed to infection. The results are summarized in table I and figure 1.

M. monax, *woodchuck*. All animals of this species became infected. The percentage recovery of adult worms was not significantly different from that of the albino mice used as

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TABLE I. *Susceptibility of wild mammals to experimental infection with* Schistosoma mansoni.

Hosts	Duration of infection (weeks)	No. of hosts examined	Mean no. of worms recovered	Percentage of worms recovered	Males : Females	Parasite measurements in mm					
						Females		Males		Females	Males
						Mean	SE ±	Mean	SE ±		
RODENTIA :											
M. monax	8–10	5	245	40	1.0	5.60	.273	5.52	.174	1.0	
P. leucopus	8–10	10	27	11	1.0	7.30	.198	5.30	.207	1.4	
M. pennsylvanicus	8–10	4	80	14	1.0	5.70	.142	6.00	.227	1.0	
S. carolinensis	8–10	4	80	38	1.1	5.30	.160	5.66	.127	1.0	
M. coypus	11–12	5	170	21	2.0	3.80	.101	3.90	.153	1.0	
T. striatus	11–12	3	47	19	1.0	3.90	.224	4.00	.164	1.2	
R. norvegicus	11–12	5	7	4	1.3	4.57	.291	3.85	.144	1.2	
O. zibethicus	11–12	6	0	—	—	—	—	—	—	—	
Z. hudsonius	11–12	3	4	2	1.0	5.93	.806	5.45	.244	1.1	
CARNIVORA :											
P. lotor	11–12	5	73	15	0.9	4.90	.177	5.34	.262	0.9	
M. mephitis	11–12	3	54	11	0.9	8.40	.335	6.43	.329	1.3	
V. fulva	11–12	3	0	—	—	—	—	—	—	—	
LAGOMORPHA :											
S. floridanus	11–12	5	18	5	0.9	2.80	.210	3.70	.162	0.7	
MARSUPIALIA :											
D. marsupialis	8–10	7	142	22	1.0	5.85	.169	4.93	.132	1.2	
Controls :											
M. musculus	8–10	50	74	37	1.1	6.82	.075	6.12	.064	1.1	

controls. Most parasites were found in the distal branches of the mesenteric venous system and appeared normally developed. Gross examination revealed ascites, marked fibrosis, enlargement and brownish discoloration of the liver, presumably due to pigmentation, numerous hemorrhages in the intestine, enlargement of the spleen, and fibrosis of the lungs. Eggs and pigment were seen in pressed preparations of the liver, intestine, spleen and lungs. The prepatent period for woodchucks was 7 to 8 weeks, after which time numerous eggs were found in the feces. These eggs were viable and the miracidia were infective.

S. carolinensis, *gray squirrel.* The recovery of schistosomes from gray squirrels was not significantly different from that of the albino mice. All squirrels became infected. Most parasites were found in the branches of the mesenteric vein in the portal vessels near the liver and appeared to be normally developed. Gross examination revealed enlargement of the liver and the spleen. No hemorrhages were found by gross examination of intestine or lungs. Eggs and pigment were seen in the liver and intestine but not in the lungs. Only pigment was seen in the spleen. The prepatent period in squirrels was 8 to 9 weeks. The eggs recovered from the liver, intestine, and feces were viable and the miracidia were infective.

M. coypus, *nutria.* The worms that developed in nutria were considerably stunted and the reproductive system was underdeveloped. Most parasites were found in the intrahepatic and in the portal vessels near the liver. A few eggs were found in the stools of one animal. Enlargement of the liver and the spleen were observed. The intestine and the lungs revealed no obvious pathologic changes. Eggs and pigment were found in the liver and intestine. Viable eggs were found in the liver and the intestine, but not in the feces. Miracidia which hatched from these eggs were not infective.

T. striatus, *chipmunk.* Most parasites were found in the intrahepatic and portal vessels near the liver. The worms in chipmunks were moderately stunted and underdeveloped. No mature eggs were found in the feces. Fibrosis and enlargement of the liver was observed, together with enlargement of the spleen. However, the intestine and the lungs looked essentially normal. Eggs and pigment were recovered in pressed preparations of the liver. Only pigment was found in the intestine and spleen. The eggs recovered from the liver were viable, but the miracidia which hatched did not infect snails.

P. leucopus, *white-footed mouse.* All animals became infected, but the yield of parasites was significantly lower than the yield from mouse controls. Most parasites were found in the intrahepatic and portal vessels near the liver, but some schistosomes were also located in the mesenteric veins. The worms were essentially normal in length and development. Marked fibrosis and focal gray discoloration of the liver were observed. Eggs and pigment were seen in the intestine, liver, and spleen. The lungs appeared essentially normal. The prepatent period in white-footed mice was approximately 8 weeks.

The eggs recovered from the liver, intestine, and feces were viable and infective.

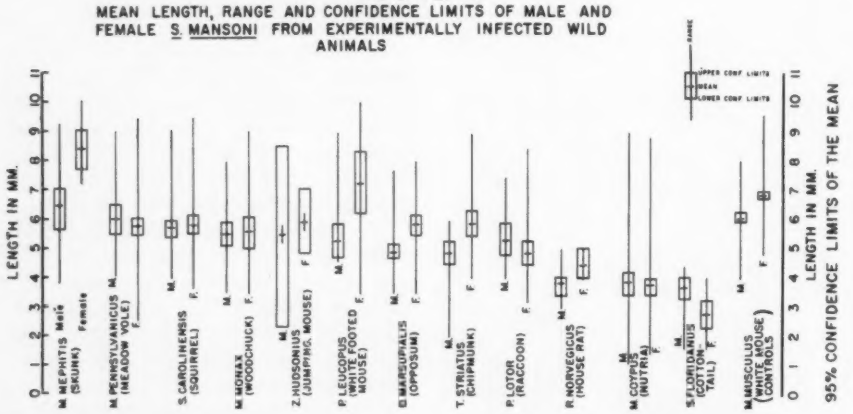
M. pennsylvanicus, *meadow vole.* The observations in all meadow voles were essentially similar to those reported for the white-footed mouse.

R. norvegicus, *house rat.* Only a few stunted worms with underdeveloped reproductive systems were recovered at necropsy in the intrahepatic veins of the five animals. No gross pathology of the liver, intestine, spleen, and lungs was observed. Some pigment and a few undeveloped eggs were found in the pressed preparations of the liver. Only pigment was observed in the intestine and spleen. No eggs were recovered from the feces at any time.

M. mephitis, *striped skunk.* The findings in all skunks were similar to those in the raccoons; the only essential difference was that the worms appeared to be considerably longer and somewhat better developed in the skunks. The length of male worms in skunks and the length ratio between females and males were not significantly different from the same measurements of worms in the albino mouse controls. The length of female worms in skunks was significantly greater than the lengths in the mouse controls. No viable eggs were recovered from the liver, and no eggs were recovered from the feces.

FIGURE 1.

MEAN LENGTH, RANGE AND CONFIDENCE LIMITS OF MALE AND FEMALE S. MANSONI FROM EXPERIMENTALLY INFECTED WILD ANIMALS



Z. hudsonius, *jumping mouse.* Only a few stunted, underdeveloped worms were recovered in the intrahepatic vessels. Fibrosis and enlargement of the liver was observed. Enlargement of the spleen also occurred. The intestine and lungs appeared essentially normal. Pressed preparations revealed eggs and pigment in the liver. The eggs recovered from the liver were viable, but the miracidia were not infective. No eggs were recovered from the feces.

O. zibethicus, *muskrat.* No schistosomes were recovered at necropsy, and all the organs appeared normal.

P. lotor, *raccoon.* Schistosomes were recovered from all raccoons. Although most of the parasites were found in the intrahepatic and portal vessels near the liver, some were also observed in the mesenteric system. The worms were somewhat stunted and underdeveloped, the

V. fulva, *fox.* No parasites were observed at necropsy in any of the three foxes experimentally exposed to infection.

S. floridanus. *cottontail.* Schistosomes were recovered in all cottontails. The worms were found in the intrahepatic veins and were markedly stunted and underdeveloped, the males being longer than the females. Eggs and focal gray discoloration were seen in the liver. The

other organs appeared essentially normal. None of the eggs recovered in the liver was viable. No eggs were recovered from the feces.

D. marsupialis, *opossum*. All opossums were infected with numerous but moderately stunted schistosomes. Although most worms were found in the intrahepatic and portal vessels near the liver, a few were located in the mesenteric system. The prepatent period in opossums was between 9 and 10 weeks. Fibrosis, brownish, discoloration, and eggs were seen in the liver. Eggs and pigment were present in the spleen. The intestine and lungs were essentially normal. The eggs recovered from the liver and intestines were viable but not infective. However, the few eggs recovered from the feces were both viable and infective.

DISCUSSION

While no *S. mansoni* infections were found in mammals trapped in the Washington, D. C.-Baltimore, Maryland, area, the laboratory experiments indicate that the parasite can establish effective infections in at least three species.

Host susceptibility varied considerably among species, although a marked uniformity of results was obtained among animals of the same species. In general, the animals tested fell into four main susceptibility groups. (1) Animals which became infected with a large number of worms and passed numerous viable eggs. The pathological findings in these animals were essentially similar to those in the controls. These animals could have epidemiological significance as potential reservoirs of infection. (2) Animals which became infected with a relatively large number of worms but whose feces contained very few viable eggs. The liver pathology in these animals was great, but little damage occurred in the intestine, spleen, and lungs. (3) Animals in which only a small number of stunted worms developed and no eggs were passed in the feces. The worms remained in the intrahepatic circulation, and the only obvious damage was in the liver. (4) Animals in which no adult worms were recovered.

The woodchuck appeared to be the most susceptible animal of all those included in this experiment, and the fox and the muskrat the most resistant. The mean length of worms recovered was usually related to the susceptibility of the host. This was particularly noticeable among female worms. The only exception was observed in the skunk where long and well-developed worms were recovered, but no viable eggs were seen.

Most rodents appeared to be more susceptible than mammals belonging to other orders. However, some species of rodents were resistant. In general, no direct correlation could be found between susceptibility and phylogeny or habitat.

In interpreting these results on the susceptibility to infection of the above mammals, attention should be called to the fact that these animals were maintained under laboratory conditions for several months. It is possible that factors such as diet and stress might have influenced the host response to infection and that these results obtained under laboratory conditions might differ from those which would be observed if the same animals acquired and developed schistosome infections in nature.

SUMMARY

Sixty-eight wild mammals of 14 species were exposed to cercariae of *Schistosoma mansoni*. Susceptibility was judged from the number of worms recovered and their location in the host, presence of eggs in the feces and various organs, viability of eggs, and the infectivity of miracidia for snails.

Susceptibility did not always follow phylogenetic criteria. The woodchuck, meadow vole, and white-footed mouse were especially susceptible to infection. The squirrel, nutria, and opossum became infected with a relatively large number of worms but only a few viable eggs were produced. Only a small number of stunted worms developed in the raccoon, chipmunk, house rat, jumping mouse, cottontail rabbit, and the skunk, but no eggs were passed in the feces. No infection occurred in the fox and muskrat.

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(Continued from Cover 4)

A DESCRIPTION OF ANOMALOUS AND ATYPICALLY DEVELOPED TAPEWORMS (PROTEOCEPHALIDAE: *Corallobothrium*) FROM CATFISHES (*Ictalurus*). **Kermit E. Sneed** (809–812)

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ERRATA: 702.

GAMETOGENESIS, EGG-CAPSULE FORMATION, AND EARLY MIRACIDIAL DEVELOPMENT IN THE DIGENETIC TREMATODE *HALIPEGUS ECCENTRICUS* THOMAS*

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Several similar species of *Halipegus* exist in the buccal cavities of frogs, and though mature forms of this genus are difficult to distinguish, species identifications can be made on the basis of differences in the size and shape of their egg capsules and on morphological differences in the miracidia and cercariae. Both mature *Halipegus eccentricus* Thomas, 1939, found primarily in the Eustachian tubes, and a species tentatively identified as *Halipegus occidualis* Stafford, 1905, found primarily under the tongue and in the esophagus, were collected from *Rana clamitans* from Oconto County, Wisconsin. Jones (1956) has found a haploid number of nine chromosomes in spermatocytes and oocytes in *H. occidualis* and suggested that this fact may be of aid in differentiating between species within this genus. The present study of *H. eccentricus* describes cellular changes in spermatogenesis, oogenesis and fertilization, formation of the egg capsule, and the early development of the miracidium, so comparisons can be made with similar stages that have been described in other monoecious trematodes.

MATERIALS AND METHODS

Egg capsules with miracidia were recovered from mature specimens of *Halipegus* which were removed from the Eustachian tubes and isolated in watch glasses with fresh water. Identifications of *H. eccentricus* were made on the basis of the size of the egg capsule which has a recurved filament about equal to the length of the main part of the capsule; on the morphology of the miracidium, distinguished partly by the presence of eight apical hooks, and by the presence of two flame cells midway between the posterior edge of the apical gland and the posterior part of the body; and on the basis of the life cycle. Laboratory raised snails of the genus *Physa* were infected by placing them with the embryonated eggs. Cercariae which were obtained from rediae were used for confirmation of the earlier identifications made on egg capsules and miracidia. Specimens used for histological purposes were fixed without compression in FAA, Bouin's, or equal parts of a saturated aqueous corrosive sublimate solution in ethyl alcohol. Sections 6 to 10 microns thick were made from these after they had been cleared in cedarwood oil and embedded in paraffin. Unless otherwise mentioned within the text, sections were stained either with iron hematoxylin and eosin or in Mallory's triple stain for general observations, with the Feulgen reaction for nuclear observations, or by the Bauer-Feulgen method for observations on glycogen. Because of possible cell displacement due to compression, the living specimens that had been used for observations on the activity within the reproductive ducts were not used for sections.

OBSERVATIONS AND DISCUSSION

Spermatogenesis. Spermatogenesis in most monoecious digenea consists of a series of mitotic divisions beginning with single primary spermatogonia and resulting in clusters of two secondary spermatogonia, four tertiary spermatogonia, and eight primary spermatocytes. Subsequently, the primary spermatocytes divide simultaneously by the first meiotic division and produce clusters of 16 secondary spermatocytes which in turn simultaneously divide by the second meiotic division and produce clusters of 32 spermatids. Each spermatid differentiates into a sperm. Earlier papers on trematode spermatogenesis have been summarized by Cable (1931), Anderson (1935), and Yosufzai (1952). More recent papers with descriptions of spermatogenesis in trematodes are those of Van der Woude (1954), Dhingra (1954 a, b; 1955 a, b, c), Guilford (1955), Perkins (1956), Ciordia (1956), Nez and Short (1957), Gresson (1958), Dunn (1959), and Burton (1960).

In *H. eccentricus* the sequence of divisions in spermatogenesis is the same but the process of sperm formation differs from that in most other species of trematodes. Primary, secondary, and tertiary spermatogonia are found adhering to the walls while intermingling clusters of spermatocytes, spermatids, and sperms float

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separately in the central portion of the testes. Primary spermatogonia, small ovoid cells with little cytoplasm, have typical interphase nuclei with one nucleolus. Adjacent secondary and tertiary spermatogonia, of the same size and appearance as the primary spermatogonia, are respectively in pairs or clusters of four. Nuclear activity was evident in most spermatogonia. Figure 1 shows spermatogonia along the walls of the testes.

Primary spermatocytes, centrally attached in clusters of eight, are found with all prophase stages of the first meiotic division. In the commonly found pachytene stages the chromosomes are polarized on one side of the nucleus around a small central nucleolus while in the clear portion of the nucleus there is unassociated nucleolar material suggesting a nucleolar extrusion such as was found in oocytes by Yosufzai (1953b) and Burton (1960). Where chromosomes were separated in the metaphase stage, 11 bivalents could be counted (fig. 2). Clusters of 16 cone-shaped secondary spermatocytes result from a simultaneous division of primary spermatocytes. Each of these undergoes a reformation of a granular nucleus before a second meiotic division. Division of secondary spermatocytes is typical of the second meiotic division and results in the formation of a cluster of 32 cone-shaped spermatids. Figure 3 shows secondary spermatocytes in the metaphase stage.

Immediately after formation the spermatids have small densely-staining telophase nuclei (fig. 4) which develop into spherical interphase nuclei (fig. 5). Sperms evolve from both the nuclei and the cytoplasm of the spermatids. Each nucleus changes into a weakly staining, nongranular, blunt, club-shaped structure (fig. 6) which progressively elongates and tapers to become a darkly-staining homogeneous sperm nucleus coiled in a plane in the central portion of a homogeneous cytoplasmic mass formed by the disappearance of the spermatid cell membranes. The cytoplasmic mass coalesces into a thick cup-shaped structure in the region where the nuclei are coiled. Long fine cytoplasmic filaments grow outward from the concave area of this mass into the fluid of the testes; these filaments eventually become the tails of the sperms (fig. 7). Then the sperm nuclei straighten simultaneously. A resulting bundle of 32 parallel sperm nuclei with the cytoplasmic filaments (fig. 8) separates from the dense cytoplasm and leaves it as a vacuolated residual mass that eventually disintegrates. In fresh material the sperms show an undulating motion with an occasional coiling or looping of the body. Motile sperms are stored in the seminal vesicle until transferred to the enlarged portion of Laurer's canal, herein called the seminal receptacle, where very active sperms in a proportionately large amount of fluid are found.

In the testes, seminal vesicle, and seminal receptacle the sperms stain a light pink with Schiff's reagent as used in Bauer's or the periodic acid-Schiff technique. After passage through Mehlis' gland the sperms in the proximal part of the uterus stain a deep red. After incubation in saliva the sperms are colorless. These results indicate the presence of small amounts of glycogen in the sperms before their passage through Mehlis' gland and an increase in glycogen after they have passed through the gland and are in the uterus. This glycogenesis probably is associated with a degeneration since sperms lose their motility and deteriorate as they proceed distally from Mehlis' gland.

Filariform sperms are found in trematodes so far described with the exception of globular sperms in *Asymphylodora* sp. (Dhingra, 1955c). In *Acetodextra amiuri* the entire spermatid elongates to form the sperm (Perkins, 1956). Dingler (1910), Woodhead (1931), Yosufzai (1952), and Burton (1960) described sperms composed of both nucleus and cytoplasm although some spermatid cytoplasm remains as a residual mass. The formation of sperms in *H. eccentricus* most closely resembles the formation of sperms in *Dicrocoelium lanceatum* as described by Dingler. Sperms with flagella exist in the hemiurid *Isoparorchis eurytremum,* in *Cyclocoelum bivesiculatum,* in *Cotylophoron elongatum,* and in *Gastrothylax crumenifer* (Dhingra, 1954 a, b; 1955 a, b) and also in the dioecious *Schistosomatium douthitti* (Nez and Short, 1957). These also have residual masses. Sperms of other trematodes are described as being entirely nuclear (Cable, 1931; Anderson, 1935; Rees, 1939; Markell, 1943; Willmott, 1950; Guilford, 1955; and Ciordia, 1956).

Oogenesis. The female reproductive system of *H. eccentricus* possesses a spherical ovary and an oviduct joined by a seminal receptacle and a vitelline reservoir. The vitelline reservoir is formed from the union of vitelline ducts leading from compact vitellaria. An ovivitelline duct

leads from the junction of the vitelline reservoir and the oviduct to an ootype in the center of a well-developed Mehlis' gland. A long uterus extends from the ootype to the genital atrium.

The ovary, except for a layer of oogonia along the distal wall, consists of primary oocytes. The oogonia, in which only occasional mitotic figures are found, are small cells with a thin layer of homogeneous cytoplasm. Small primary oocytes, though similar in size, can be distinguished from the oogonia by their nuclei which have early polarized prophase stages of the first meiotic division. As the primary oocytes and their nuclei enlarge the pachytene stages are replaced by vesicular nuclei with threadlike chromosomes or diffuse chromatin and a large nucleolus. The nuclei of the primary oocytes, which fill the central part of the ovary and the portion nearest the oviduct, retain this appearance until after encapsulation and sperm penetration and the egg is in the proximal portion of the uterus. Figure 9 shows a portion of the wall of the ovary with oogonia and young primary oocytes.

Primary oocytes within the egg capsules in the most proximal portion of the uterus are found with single attenuated sperm nuclei within their cytoplasm. Other coiled sperms occasionally were found among but not within the cytoplasm of vitelline cells in the egg. The sperm nucleus within an oocyte retains its attenuated structure until after the chromosomes of the oocyte have condensed into the diplotene stage (figs. 11, 12). By metaphase I (fig. 13) the sperm nucleus becomes a small, dense, club-shaped structure and it remains thus until after the second meiotic division of the oocyte, whereupon a male pronucleus is formed (fig. 16).

The maturation divisions of the oocyte are completed when the egg is in the proximal region of the uterus. In the diplotene stage and the prometaphase stage of the first meiotic division, 11 bivalents were found in the oocytes (figs. 12, 13). Figures 14 and 15 show the first meiotic division and formation of the first polar body. The second meiotic division occurs without reorganization of the oocyte nucleus and results in the formation of a second polar body. A female pronucleus develops concurrently with the male pronucleus; they are approximately equal in size (fig. 16). In hematoxylin stained sections a large nucleolus is found in each. The chromosomes condense and, following the disappearance of the nuclear membranes, the chromosomes of each pronucleus become arranged on the metaphase plate of the first cleavage division (fig. 17).

While the sequence of oocyte development and polar body formation is essentially similar in the monoecious trematodes in which it has been described, some, e.g., *H. eccentricus*, form the metaphase of the first cleavage division immediately after the condensation of the chromosomes in a prophase stage in each pronucleus (Rees, 1939; Jones, Mounts, and Wolcott, 1945; Willey and Koulish, 1950; Guilford, 1955; and Dhingra, 1954a). In others the pronuclei are said to fuse and form an interphase zygote nucleus prior to the first cleavage division (Chen, 1937; Yosufzai, 1953a; Willmott, 1950; Van der Woude, 1954; and Dhingra, 1955c).

In fresh material the oocytes of *H. eccentricus* are filled with refractile granules which apparently are extracted in embedding techniques. Darkly staining bodies, interpreted as food material, that occur in most other trematodes, were not found in this species nor could the presence of glycogen in the cytoplasm of the oocytes be demonstrated with the Bauer-Feulgen technique; this agrees with the work of Axmann (1947) who found little or no glycogen in the oocytes of a variety of trematodes.

Chromosomes. Eleven bivalents; 1 large, 3 medium, and 7 small; are found in the primary oocytes and spermatocytes of *H. eccentricus* (figs. 2, 13). Two long, 6 intermediate, and 14 small chromosomes make up the diploid set of 22 found in zygotes (fig. 17). It is thus possible to distinguish *H. eccentricus* from *H. occidualis* on the basis of chromosome number since Jones (1956) reported n = 9 for the latter species.

Vitelline cells and formation of egg capsule. Developing and fully developed vitelline cells surrounded by a thin fibrous layer make up the compact vitellaria of *H. eccentricus*. The smallest vitelline cells, found near the periphery of the follicles, have a thin layer of homogeneous cytoplasm. Progressing inward from these to a sharply defined central zone of fully developed cells, there is an area of growing cells in which globules of shell material accumulate, fuse, and increase in size within the cytoplasm while the nuclei remain unchanged. In mature vitelline cells globules may become as large as the nucleus. Figure 10 shows vitelline cells in

different stages of development. The development of shell material, from its first appearance as small foci within the cytoplasm until it is released and converted to shell capsule was observed easily in tissue stained with Mallory's triple stain because the vitelline globules stain bright red and the surrounding material blue. With the methods employed globules of shell material appeared to be homogeneous.

Egg formation occurs during a coordinated sequence of contractions in the proximal ducts of the female reproductive system. Contractions in the fibromuscular wall at the hilum of the ovary compress and force mature oocytes into the proximal oviduct where a line of oocytes is retained by a sphincter. Periodically the sphincter opens and permits one oocyte into the more distal part of the oviduct where it is forced by muscular contractions to the opening of the seminal receptacle. At this time the seminal receptacle contracts and its sphincter opens discharging sperms and fluid into the oviduct behind the oocyte. The discharge of fluid pushes the oocyte surrounded by sperms to the junction of the ovivitelline duct just distal to the opening of the vitelline reservoir. Body contractions that compress the vitellaria and peristaltic contractions of the vitelline ducts push a continuous line of fully developed vitelline cells into the vitelline reservoir where intact cells remain until it contracts and discharges six to nine of the vitelline cells into the ovivitelline duct behind the oocyte. By a contraction of the ovivitelline duct this group of cells, oocyte first, is carried to the ootype where the shell material is suddenly released from the vitelline cells. Rhythmic contractions of the ootype distribute the material around the vitelline cells and forward around the oocyte.

Figure 11 shows a section with an egg capsule in formation within the ootype. Just after formation the outermost shell material is evenly distributed along the wall of the ootype in the region of the egg, but within the capsule it is irregularly distributed among the enclosed cells. Pressure from contractions of the ootype and later from the narrow portion of the uterus compresses the pliable shell material against the cells within, and by this means redistributes the shell material into a nonstratified capsule of uniform thickness. The posterior filament of the capsule is formed when the released shell material is constricted in the ovivitelline duct as peristaltic waves of the ootype and the most proximal portion of the uterus force the main part of the capsule forward. The shell material becomes tanned and less pliable as the egg moves distally in the uterus. Only one egg is formed at a time and the sequence is not repeated until the newly formed egg is pushed into the narrow proximal portion of the uterus. In four living but compressed specimens, 20 to 30 seconds elapsed from the time an oocyte was released by the sphincter in the oviduct until the egg was forced from the ootype into the uterus.

The globules within the vitelline cells and the pliable material of the newly formed shell have the same staining reactions. They turn reddish brown in Millon's reagent and stain blue in brom-phenol blue and in methylene blue, which shows a protein content; they turn brown with incubation in catachol which shows the presence of phenolase. These findings indicate that the capsule probably becomes quinone-tanned sclerotin such as that described in *Fasciola hepatica* and several other trematodes by Stephenson (1947) and Smyth and Clegg (1959). In fully developed vitelline cells the cytoplasm surrounding the globules of shell material stains red with Schiff's reagent as used in Bauer's method or the periodic acid-Schiff reaction, while after 1 hour of incubation in saliva the cytoplasm remains unstained, indicating the formation of glycogen in the cytoplasm of fully developed vitelline cells. In the central zone of the vitellaria and the area leading to the vitelline ducts mature vitelline cells are sharply delineated by the presence of glycogen in their cytoplasm, from the developing vitelline cells nearer the periphery. After capsule formation the glycogen content of the cytoplasm appears to be the same. Glycogen has been reported in the vitelline cells of other trematodes by Axmann (1947), Rao (1959), and Govaert (1960).

Early development of the miracidium. The miracidium of *H. eccentricus* as described by Thomas (1939) is a simple one with a spiny cuticle, a large apical gland with one anterior and five postero-lateral pairs of glands, two flame cells in the mid-posterior region of the body, and a cluster of germinal cells in the posterior part of the body. Since the miracidium develops while the eggs are in the uterus, early developmental stages were available for exami-

nation and comparison with the few miracidia for which the development is known.

In this species the zygote is closely confined to the anterior portion of the egg so the embryo grows posteriorly into the degenerating vitelline cells and eventually fills the capsule. The first cleavage division is unequal, the anterior cell being slightly larger. In the two-cell stage mitotic figures were found only in the anterior cells (fig. 18) but in succeeding 3-, 4-, or 5-cell stages figures were found in the other cells also. Figure 19 shows a 4-cell stage with mitosis in the posterior cell.

An unequal first cleavage division has been described in other trematodes, and in recent papers the anterior cell has been referred to as an ectodermal cell. In these trematodes this cell divides a number of times and the descendants produce much of the somatic tissue. The posterior cell is referred to as a propagatory cell and remains distinct from the somatic antecedents in the early stages. Descendants of the propagatory cell produce cells of only the germinal line (Dunn, 1959) or of the germinal line and in addition some of the somatic tissue (Ishii, 1934; Chen, 1937; Rees, 1940; Van der Woude, 1954; Guilford, 1958). Dhingra (1955c) reported a segregation of a propagatory cell in the early embryo of *Asymphylodora* sp. and Willey and Godman (1951) found an uneven cleavage in *Zygocotyle lunata* and traced the large undividing cell at the end of the embryo through a 5-cell stage, but the latter authors were unable to confirm that the large cell was an early segregation of the germinal line.

Though early segregation of a germinal line is not evident in *H. eccentricus*, the differentiation of the somatic cells of the miracidium parallels the sequence of development in other miracidia. Vitelline membrane cells are the first to differentiate. The most posterior cell of the 7- or 8-cell stage, which may remain attached or may separate from the embryo, ceases to divide and becomes a cup-shaped cell with a pyknotic nucleus at the posterior end of the embryo (fig. 20). Later in the 15- to 20-cell stage an anterior vitelline membrane cell segregates and becomes a cap-like cell over the anterior end of the embryo (fig. 21, 22). A similar early differentiation of vitelline membrane cells has been described in miracidial embryos by Ortmann (1908), Ishii (1934), Chen (1937), Rees (1940), Van der Woude (1954), Guilford (1958), and Dunn (1959). The two vitelline membrane cells in *H. eccentricus* represent a reduced number of these cells when compared with other trematodes.

The miracidial embryo develops with little further differentiation until it is composed of from 40 to 60 cells; internal structures then become evident and thereafter only a slight increase occurs in the number of cells. Figure 22 shows the differentiation in a miracidium with about 60 cells. By the 50-cell stage the epidermal cells are easily recognizable by their dense nuclei around the periphery of the embryo. These change into a Bauer-Feulgen positive tunic instead of differentiating into tiers of ciliated cells as is common in other trematodes. A subepidermis is evident beneath the epidermis. The apical gland (also referred to as a primitive gut) develops from the fusion of two large anterior cells which can be distinguished by their large nuclei with nucleoli and by an enlarging mass of clear cytoplasm. Behind the apical gland and within the subepidermis there is a compact mass of cells that subsequently develop into flame cells, germinal cells, and lateral glands which stain deep red in the Bauer-Feulgen technique. A nervous system could not be identified in the embryo.

SUMMARY

The stages of spermatogenesis, oogenesis, capsule formation, and early miracidial development were studied in *Halipegus eccentricus*.

The two testes contain spermatogonia, spermatocytes, spermatids, and sperms. A sequence of three spermatogonial divisions and two meiotic divisions terminates in the formation of a cluster of 32 spermatids; the spermatids differentiate into sperms with elongate nuclear heads and cytoplasmic tails, and a residual mass of cytoplasm. Sperm nuclei within oocytes condense into rod-shaped structures before forming a male pronucleus.

The ovary consists of a peripheral layer of oogonia, young oocytes with early synaptic stages and a central part composed of enlarged oocytes with vesicular nuclei. The oocyte retains this form until after encapsulation, penetration by a single sperm, and until the egg is in the proximal part of the uterus; there it undergoes diakinesis and two meiotic divisions.

A haploid chromosome number of 11 was found in spermatocytes and oocytes, and a di-

ploid number of 22 in zygotes.

Vitelline cells, formed in vitellaria, have globules of material that form an unstratified egg capsule in the ootype. Encapsulated vitelline cells disintegrate as the miracidial embryo fills the egg capsule.

Glycogen was found in fully developed vitelline cells and in sperms, but not in oocytes.

The simple miracidium of *H. eccentricus* does not show an early differentiation into somatic and propagatory cells, but the development of somatic tissues, i.e., the differentiation of the vitelline membrane cells, epidermal and subepidermal cells, and the development of glands, occurs in a sequence similar to that in the miracidia of other trematodes.

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EXPLANATION OF PLATE I

Abbreviations: AG, apical gland; E, epidermal cell; G, germinal cell; S, sperm; V, vitelline membrane cell. Figures were made with the aid of camera lucida.

FIGURE 1. Spermatogonia near the periphery of a testis.

FIGURE 2. Primary spermatocytes in metaphase I.

FIGURE 3. Secondary spermatocytes during the second meiotic division.

FIGURE 4. Spermatids after division of secondary spermatocytes.

FIGURE 5. Spermatids with interphase nuclei.

FIGURE 6. Spermatids with elongating nuclei.

FIGURE 7. Sperm formation with nuclear heads coiled in the cytoplasmic mass.

FIGURE 8. Cluster of sperms after separation from the cytoplasmic mass.

FIGURE 9. Periphery of the ovary showing developing primary oocytes.

FIGURE 10. Stages in development of vitelline cells.

FIGURE 11. Egg formation in the ootype. A primary oocyte with a sperm nucleus and 4 vitelline cells enclosed in a newly formed capsule in which shell material is unevenly distributed.

FIGURE 12. Diakinesis in a primary oocyte.

FIGURE 13. Prometaphase in a primary oocyte showing 11 chromosomes and a sperm nucleus.

FIGURE 14. Early anaphase I of the oocyte.

FIGURE 15. Formation of the first polar body.

FIGURE 16. Formation of the second polar body: Male and female pronuclei are within the oocyte.

FIGURE 17. Zygote with a diploid number of chromosomes.

FIGURE 18. Two-cell stage of miracidial embryo just prior to a second cleavage division.

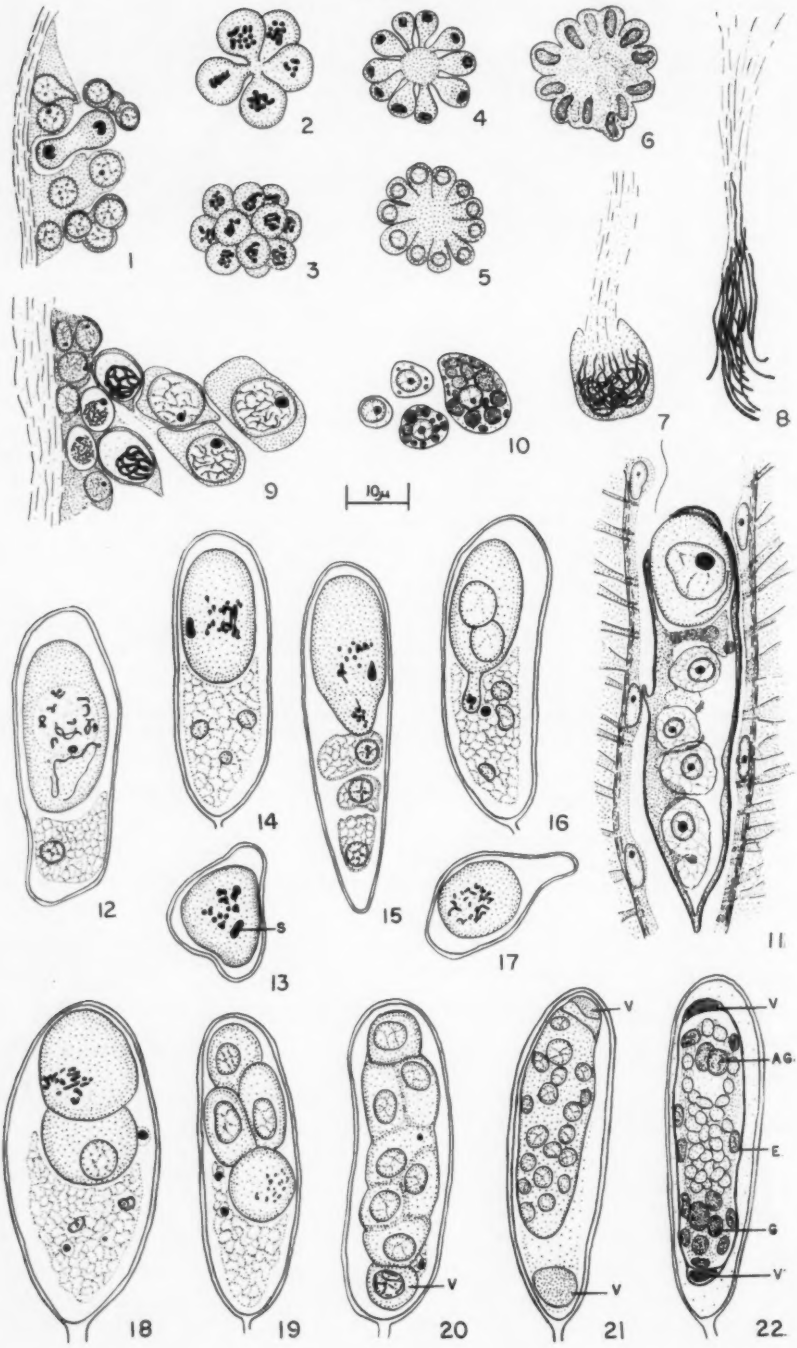
FIGURE 19. Four-cell stage of a miracidial embryo showing mitotic activity in the posterior cell.

FIGURE 20. Eight-cell stage of a miracidial embryo showing the posterior vitelline membrane cell.

FIGURE 21. Twenty-seven cell miracidial embryo with anterior and posterior vitelline membrane cells.

FIGURE 22. Miracidial embryo of about 60 cells showing the differentiation of the apical gland, epidermal cells, and germinal cells.

PLATE I



TREMATODES OF FISHES FROM THE RED SEA. PART 9. SIX NEW ANAPORRHUTINE SPECIES INCLUDING A NEW GENUS

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The trematodes described below were collected by the senior author at the Marine Biological Station at Ghardaga, Red Sea. Type specimens are deposited in the Department of Parasitology of Faculty of Medicine, Abbassia, Cairo, Egypt., U. A. R.

All measurements are in millimeters.

Family GORGODERIDAE

Subfamily ANAPORRHUTINAE

Anaporrhutum mantae n. sp. (Fig. 1)

Description based on whole mounts of three of five specimens from *Manta ehrenbergi* locally called "Manta;" probably in body cavity; large, translucent worms, 40 to 44 long, 21 to 27 in greatest width. Cuticle lacking scales or spines with two depressions on either side of oral and ventral suckers. Oral sucker terminal 1.4 to 1.6 in diameter. Pharynx strongly muscular, overlapping oral sucker posterodorsally, 0.6 to 0.8 in diameter. Ceca unbranched, undulating, terminating 1.4 to 2.1 from posterior end of body. Ventral sucker 1.2 to 1.5 in diameter, 0.4 to 1.0 from oral sucker.

Two lateral sets of testicular follicles 0.2 to 0.3 in diameter, in second and third fifth of body length, partly inter- and partly extracecal with fewer follicles between ceca; left set with average of 24, right 19. Long axis of testicular follicles parallel with long axis of trematode.

Female complex almost in one plane between ceca at posterior end of first quarter of body length. Ovary 0.3 to 0.4 in diameter, ovoid; receptaculum seminis anterolateral to ovary, spherical, 1.3 to 1.5 in diameter, 1.8 to 3.4 from ventral sucker. Vitellaria consisting of two finely dendritic structures lateral to receptaculum seminis, with 19 to 20 branches on right, 25 to 28 on left. Uterus narrow, extending almost to posterior end and then anteriorly to uterine pore, situated posterior to intestinal bifurcation. Eggs oval, operculated, 0.08 by 0.09. Excretory vesicle Y-shape, each of its arms receiving an extracecal anterior and posterior excretory tubule.

Comparisons. Anaporrhutum mantae is closest to *A. albidum* Ofenheim, 1900, but differs from that species in having: (1) vitellaria intercecally situated rather than ventral to the intestinal ceca; (2) the long axis of testicular follicles parallel with that of the body instead of oblique; (3) body much larger in size; and (4) eggs also are much larger.

Nagmia nebrii n. sp. (Figs. 2 and 2a)

Description based on whole mounts of 8 of 11 preserved specimens from the body cavity of the fox or thresher shark, *Nebrius concolor;* habitat not known. Body often folded and wrinkled 13.4 to 20.6 long and 10.9 to 18.1 in greatest width; posterior part broad, rounded, and sometimes notched. Cuticle without scales or spines. Oral sucker terminal, measuring 1.1 to 2.2 in diameter; pharynx 0.4 to 0.6 strongly muscular, may overlap oral sucker posterodorsally. Espohagus 0.1 to 1.1 long. Intestinal ceca corrugated, terminating 0.8 to 1.9 from posterior end of body. Ventral sucker 2.4 to 3.8 in diameter, in second quarter of body length; 1.1 to 4.4 from oral sucker. Ratio of oral to ventral sucker 1: 2.

Testes extracecal, each group consisting of a mass of irregularly arranged follicles 0.2 to 0.3 in diameter, usually at different levels on the two sides, nearer intestinal ceca than lateral body margin, in second third of body length. Left testis with 4 to 23, right with 9 to 15 follicles.

Female complex in second third of body length and slightly varying in different specimens. Ovary entire, ovoid, 0.3 to 0.6 in diameter, close and posterolateral to spherical receptaculum seminis with a diameter of 0.8 to 1.3. Part of ovary and receptaculum seminis may overlap ventral sucker posteriorly. Vitellaria intercecal, in two nearly symmetrical dendritic masses, lateral to ovary and receptaculum seminis. Masses on left with 6 to 9 tubules, on right with 5 to 7. Uterus a narrow, coiled, intercecal tube, extending almost to posterior end, and then anteriorly to open just posterior to pharynx. Eggs oval, 0.06 by 0.03.

Comparisons. N. nebrii resembles *N. yorkei* Nagaty, 1930, but differs from that species in having: (1) testicular follicles irregularly arranged, fewer in number, nearer intestinal ceca than lateral body margin; (2) ovary lying lateral to receptaculum seminis instead of being posterior; (3) vitellaria differing in shape of tubules and their position relative to ovary and receptaculum seminis; and (4) uterus with

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shorter lateral coils, and the genital pore just posterior to pharynx instead of being posterior to intestinal bifurcation. It differs from *N. floridensis* Markell, 1953, mainly in having a more anterior genital pore, and the receptaculum seminis is spherical and regular in outline. It differs from *N. rosettensis* Melouk, 1940, mainly in having (1) ovary ovoid instead of pear-shaped and (2) vitellaria of intercecal, dendritic tubules, instead of poorly dendritic, overlapping ceca.

Nagmia stegostomatis n. sp. (Figs. 3 and 3a)

Description based on whole mounts of 12 of 16 preserved specimens from liver of tiger shark, *Stegostoma tigrinum*, locally called "Franca," habitat unknown. Worms often folded, wrinkled and difficult to mount, 8.1 to 9.7 long, 5.5 to 6.3 wide, posterior region broad, sometimes notched. Cuticle without spines or scales. Oral sucker terminal 0.7 to 1.1 in diameter; pharynx 0.3 to 0.5, may overlap oral sucker posterodorsally. Esophagus 0.1 to 0.5 long; ceca corrugated terminating 0.6 to 0.8 from posterior end of body. Diameter of ventral sucker 1.3 to 2.1; ratio of oral to ventral suckers nearly 1: 2; distance between them 0.9 to 2.1.

Testes extracecal, each group consisting of 15 to 17 follicles averaging 0.1 in diameter, situated close to posterior end of second third of body length, at about same level, nearer ceca than lateral margin of body.

Female complex near midlevel of body. Ovary 0.1 to 0.2 in diameter, usually composed of two separate coffee-bean-shaped lobes with a minute lobe between, in close contact with and lateral to spherical receptaculum seminis 0.3 to 0.5 in diameter and sometimes overlapping posterior border of ventral sucker. Vitellaria intercecal, consisting of two sets of 6 to 9 coarsely dendritic tubules posterolateral to ovary and receptaculum seminis. Uterus an intercecal, coiled, narrow tube extending first almost to posterior end, then anteriorly to open at uterine pore just posterior to pharynx. Eggs oval, operculated, averaging 0.05 by 0.03.

Comparisons. N. stegostomatis resembles *N. nebrii* but differs from that species in having: (1) smaller sized body; (2) testicular follicles more regularly arranged and greater in number; (3) ovary composed of two coffee-bean-shaped lobes with a minute lobe between instead of a single ovary.

Pernagmia nebrii n. g., n. sp. (Figs. 4 and 4a)

Description based on whole mounts of 12 of 15 preserved specimens from coelom of *Nebrius concolor*. Worms often folded, wrinkled and difficult to mount; 10.3 to 11.3 long, 10.3 to 11.8 wide, posterior region broad, rounded, may be notched. Cuticle without scales or spines. Oral sucker terminal, 1.1 to 1.6 in diameter, prepharynx absent; pharynx strongly muscular 0.3 to 0.7 in diameter. Esophagus 0.3 to 0.7 long. Intestinal ceca irregularly branched, terminating 0.2 to 1.7 from posterior end of body. Ventral sucker 1.9 to 3.2 in diameter; ratio of oral to ventral sucker nearly 1: 2; distance between them 2.0.

Testes two sets of extracecal follicles occupying an elongated area, very close to ceca, at different levels, usually more posterior on right, near junction of middle and posterior third of body length; follicles subspherical 0.2 to 0.3 in diameter; 13 to 15 follicles on left, 17 to 19 on right.

Female complex at midlevel of body; ovary oval, 0.2 to 0.3 in diameter, in close contact with and lateral to spherical receptaculum seminis 0.5 to 0.8 in diameter; the two structures may overlap ventral sucker posterodorsally; vitellaria consist of two compact intercecal groups each with 13 to 17 follicles, 0.12 to 0.14 in diameter; right vitelline lobe may be closer than left to ovary and receptaculum seminis. Uterus a compact, coiled tube extending almost to posterior end of body in some specimens, and filling intercecal space in posterior half of body length, anteriorly reaching to uterine pore just posterior to pharynx. Eggs oval; operculated, averaging 0.06 by 0.03.

Pernagmia concolori n. sp. (Figs. 5 and 5a)

Description based on a whole mount of single specimen from the coelom of *Nebrius concolor*. Body 14.7 long, 8.8 wide; posterior region broad, notched. Cuticle devoid of scales or spines, much folded. Oral sucker terminal 1.6 to 1.7; pharynx strongly muscular, 0.7 by 0.8 in diameter. Esophagus 1.2 by 0.4 long. Intestinal ceca corrugated, ending 1.1 from posterior end of body. Ventral sucker 2.9 by 3.7, overlapping intestinal ceca; ratio of oral to ventral sucker 1: 2; distance between them 3.12.

Testes extracecal, about symmetrical, at middle of third quarter of body length; consisting of an elongated zone of follicles on each side, 14 on left, 15 on right, close to ceca; follicles spheroid, 0.27 to 0.29 in diameter.

Female complex at midlevel of body; ovary oval, 0.5 by 0.4, in close contact with subspherical receptaculum seminis 1.0 by 0.9, the two overlap ventral sucker posteriorly. Vitellaria of two sets of 15–17 intercecal lobes nearly at same level, immediately posterolateral to ovary and receptaculum seminis; lobes average 0.09 by 0.12. Uterus a compactly coiled tube, filling the intercecal space almost to posterior end of body, anteriorly reaching to uterine pore just posterior to pharynx. Eggs oval, operculated, averaging 0.07 by 0.03.

Comparisons. P. concolori closely resembles *P. nebrii* but differs from it in (1) size of body, (2) much longer esophagus and (3) vitellaria differing in shape and their position relative to ovary and receptaculum seminis.

Pernagmia stegostomatis n. sp. (Figs. 6 and 6a)

Description based on whole mount of 8 of 12 specimens from liver of the tiger shark, *Stegostoma tigrinum* locally called "Franca." Body 14.5 to

16.4 long, 10.5 to 15.8 wide; posterior end somewhat broad, may be notched. Cuticle without scales or spines, with many folds. Oral sucker terminal 1.2 to 2.1 in diameter; pharynx strongly muscular, overlapping posterior part of oral sucker, 0.4 to 0.8 in diameter. Esophagus a narrow tube 0.4 to 1.0 long. Ceca corrugated, ending 1.2 to 1.5 from posterior end of body. Ventral sucker 2.5 to 3.7 in diameter, ratio of oral to ventral sucker 1: 2; distance between them 1.1 to 2.

Testes extracecal consisting of two sets of follicles 0.2 to 0.4 in diameter, 17 follicles in left set, 16 in right; at the same or different levels, close to ceca.

Female complex in anterior part of second third of body length; ovary oval, 0.3 to 0.7 in diameter in close contact with and lateral to spherical receptaculum seminis 0.6 to 1.6 in diameter. The two may overlap ventral sucker posteriorly. Vitellaria consisting of two sets of intercecal follicular masses lateral to ovary and receptaculum seminis, nearly at same level. Groups of follicles on each side form branch-like clusters consisting of 35 follicles on left, 30 on right. Uterus a coiled intercecal tube, not quite reaching posterior end of body; anteriorly extending to uterine pore just posterior to pharynx. Eggs oval averaging 0.06 by 0.03.

Comparisons. P. stegostomatis resembles *P. nebrii* but differs in having: (1) testes less regular in outline and (2) vitellaria of greater number of small follicles, collected on each side in groups forming branch-like clusters instead of being compact groups.

Pernagmia

DIAGNOSIS: Gorgoderidae. Anaporrhutinae. Large distomes with posterior end rounded, sometimes notched. Oral sucker terminal, pharynx well developed, esophagus short or long, ceca with irregular outpocketings, terminating blindly near posterior end. Ventral sucker moderately large. Testes consist of numerous regularly shaped, extracecal follicles very close to ceca. Ovary postacetabular; receptaculum seminis present. Vitellaria consisting of two intercecal follicular groups. Genital pore midventral, just posterior to pharynx.

Pernagamia resembles closely the genus *Nagmia*, from which it differs mainly in the shape of the vitellaria which are composed of follicles in *Pernagmia* rather than tubular masses as in *Nagmia*.

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EXPLANATION OF PLATES

PLATE I

Figure 1. *Anaporrhutum mantae*, n. sp.
Figure 2. *Nagmia nebrii* n. sp.
Figure 2a. *Nagmia nebrii* genitalia.
Figure 3. *Nagmia stegostomatis* n. sp.
Figure 3a. *Nagmia stegostomatis* genitalia

PLATE II

Figure 4. *Pernagmia nebrii* n. g., n. sp.
Figure 4a. *Pernagmia nebrii* genitalia
Figure 5. *Pernagmia concolori* n. sp.
Figure 5a. *Pernagmia concolori* genitalia
Figure 6. *Pernagmia stegostomatis* n. sp.
Figure 6a. *Pernagmia stegostomatis* genitalia

PLATE I

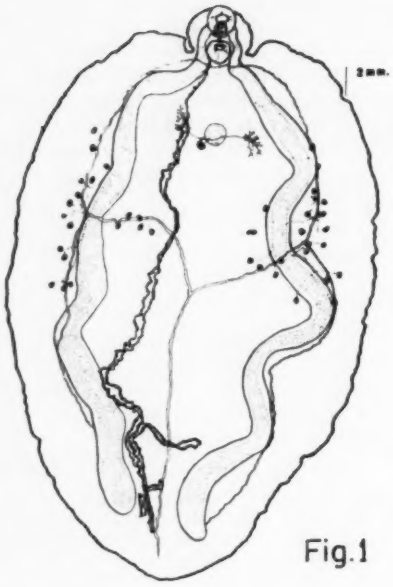

Fig.1

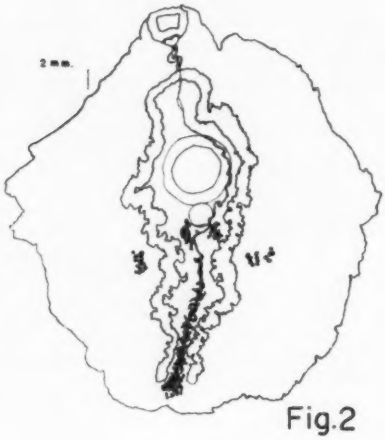

Fig.2

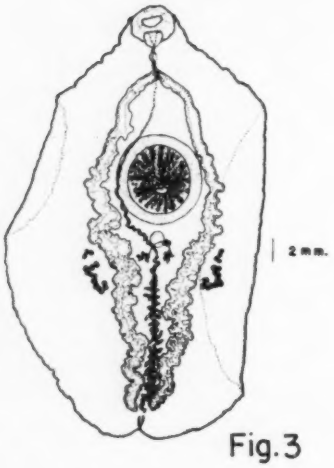

Fig.3

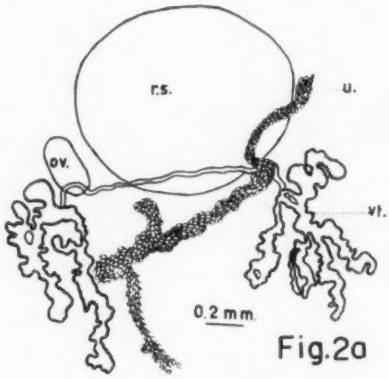

Fig.2a

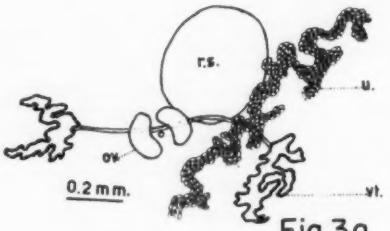

Fig.3a

PLATE II

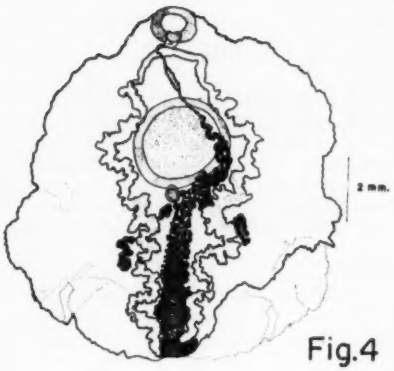

Fig.4

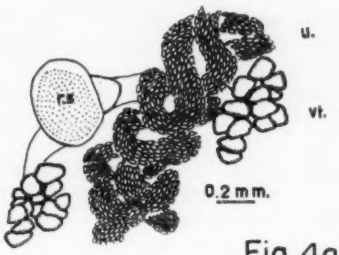

Fig. 4a

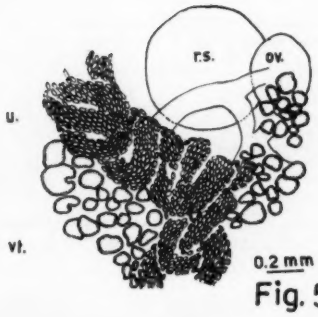

Fig. 5a

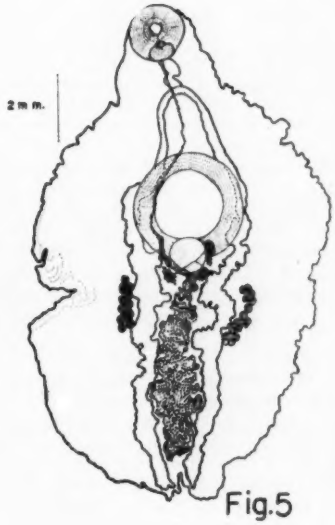

Fig.5

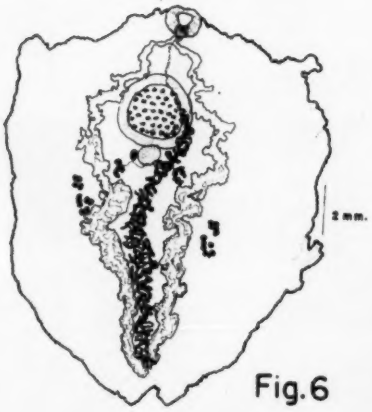

Fig.6

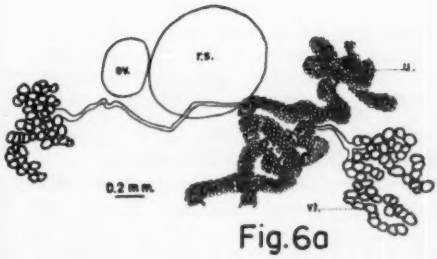

Fig.6a

STUDIES ON LARVAL *FASCIOLOIDES MAGNA*. III. MASS HATCHING OF MIRACIDIA BY EXPOSURE TO NITROGEN

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In a brief earlier report (Friedl, 1960), a convenient way of obtaining miracidia of *Fasioloides magna* (Bassi, 1875) Ward, 1917, from embryonated eggs exposed to a partial vacuum or to cylinder nitrogen at atmospheric pressure was noted. Improved methods of handling these eggs and an experiment demonstrating the efficacy of the nitrogen treatment are presented in this paper.

MATERIALS AND METHODS

Source material for the experiment was obtained in November 1957 from fluke-infected livers of white-tailed deer (*Odocoileus virginianus borealis* Miller) in Minnesota. Adults of *F. magna* are found within enlargements of the bile passages. Eggs recovered by washing slices of liver in water were strained and then sedimented several times until freed of debris. They were stored in bottles or flasks (of 100 to 200 ml volume) under 2 to 5 cm of water at 5 to 10 C with no special aeration provided. Distilled water was preferred because chlorinated tap water apparently decreased the viability of the eggs. A mat of fungus growth, commonly developing under the above storage conditions, was broken up and partially removed at intervals by re-sedimentation.

To secure miracidia for test hatching, several thousand eggs that had been stored for 2 years were transferred to a 30 ml vial containing 5 ml of SX salt solution (table I) and stoppered with a perforated plastic cap (fig. 1a). Here in an approximately single layer on the bottom of the vial, kept in the dark at 21 C, the eggs were found to embryonate favorably within a few weeks.

In the present experiment, the eggs were resuspended at the end of 5 weeks, and two 2-ml aliquots pipetted into new vials; a 0.5-ml aliquot was set aside to be used for egg counts. Specially prepared stoppers (fig. 1b) were employed with each vial, one being connected by tubing to a cylinder nitrogen supply (Ohio Chemical and Surgical Co. Water-pumped Nitrogen; specified 99.5 percent pure), and the other to a laboratory compressed air outlet. These stoppers not only permitted the egg-containing vials to have their nitrogen and air content changed, but an attached syringe allowed small samples (0.05 ml) of fluid to be removed at will. The desired gases were bubbled through water (about 95 bubbles per

TABLE I. *Constitution of SX salt solution in which* F. magna *eggs were embryonated.**

SALTS	MG PER LITER
NaCl	1000
KCl	50
Na_2HPO_4	25
$MgSO_4 \cdot 7H_2O$	100
$CaCl_2$	87
$NaHCO_3$	120
Disodium EDTA**	25
TRACE ELEMENTS* **	**MICROGRAMS PER LITER**
$CUSO_4 \cdot 5H_2O$	10
$FeCl_3$	100
H_3BO_3	100
$MnSO_4 \cdot 4H_2O$	100
$ZnCl_2$	10

* Phenol red, 2.5 mg per liter, is ordinarily added as a pH indicator.
** Disodium ethylenediamine tetraacetate.
*** Conveniently supplied from a 1000 × stock solution.

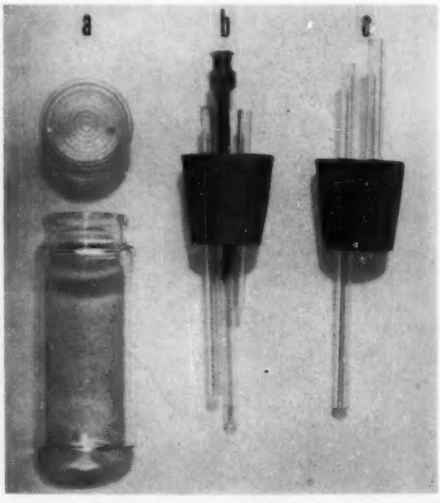

FIGURE 1. Container and stoppers used in handling *F. magna* eggs. See text.

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minute) before entering and after leaving each vial. The experiment was performed without special agitation of the vials which were kept in a water bath at a temperature of 25 C and were not shielded from the light of the laboratory. At the stated intervals, samples were removed and swimming miracidia counted. From these counts, estimates were made of the total number of miracidia that appeared.

Similar methods, used to obtain large numbers of miracidia routinely, employed as an alternative a stopper without the sampling modification (fig. 1c).

RESULTS

The total number of swimming miracidia present in samples exposed to nitrogen and to air are plotted in figure 2. Each vial contained approximately 4,400 eggs, about half of which were embryonated at the time. The lot subjected to nitrogen produced an estimated maximum of 1100 swimming miracidia in 8 hours, i.e., half of the embryonated eggs had hatched; in the vial flushed with compressed air no free miracidia were observed. It was of interest to note that under continuation of these conditions, (i.e., constant 25 C temperature, light exposure and nitrogen) activity of the free-swimming miracidia in the salt solution began to decline after the eighth hour (as observable in fig. 2, line A) at which time immotile forms were also present in the samples.

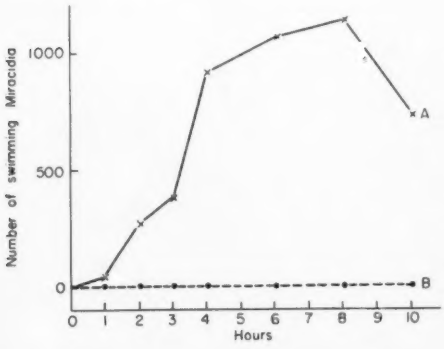

FIGURE 2. Illustrative experimental results of active miracidia of *F. magna* obtained after inducing hatching under conditions of constant 25 C temperature, in the light, and exposed to nitrogen (A, full line), as contrasted with exposure to air (B, dash line) under parallel conditions. The latter embryonated eggs, failing to hatch when exposed initially to air only (B), began hatching when exposed to nitrogen 24 hours later. (See text).

Twenty-four hours later, at 25 C under similar conditions, the contents of the vial previously exposed only to air during the test period were flushed with cylinder nitrogen. This resulted in the hatching at this time of about one-fourth of the number of embryonated eggs it had originally contained.

The above result has been reproducible substantially at will and this method has been used to advantage to secure miracidia for various experimental purposes.

DISCUSSION

The feasibility of storing large numbers of *Fascioloides magna* eggs for long periods and subsequently obtaining normal miracidia in large numbers when wanted is apparent. It may be noted that half of the eggs stored for 2 years would produce miracidia. Fungus growths, affecting the viability of stored eggs in varying degrees, have been bothersome, but their suppression thus far has not been accomplished by any simple measure taken against them.

The SX salt solution employed has been found to provide favorable conditions for embryonic development and embryonated eggs that have been stored in it for as long as 8 weeks in the dark at 21 C are capable of hatching. The number of swimming miracidia obtained from various samples of eggs has not been thoroughly investigated; however, under similar conditions, nitrogen exposure has induced hatching of up to 80 percent miracidia-containing eggs.

Immediate exposure to nitrogen in the present experiment resulted in eclosion of about half of the embryonated eggs. In the control, subjected to nitrogen after 24 hours, a count of swimming miracidia indicated that about one quarter of the original number of eggs estimated to be embryonated hatched at this time. This response reinforced evidence for the effect of nitrogen exposure and confirmed the viability of the control group.

From previous work, it appears that the total harvest of miracidia may be dependent upon a variety of factors, including the particular host from which the eggs were secured, the extent of contamination during storage, and the conditions of embryonation, as well as upon the adequacy of the exposure to nitrogen and light. In a few instances, samples of embryonated eggs have been refractory to hatching with ni-

trogen exposure. Light itself, under certain circumstances can apparently induce mass hatching, but at times it has proved inadequate in the absence of other stimulation. Conversely, although nitrogen exposure has been effective in subdued light, it is not always completely so.

Of interest are the parallel effects of partial vacuum (Friedl, 1960) and cylinder nitrogen as hatching stimuli. On the one hand, the atmosphere is being removed, and on the other it is being altered. Partial anaerobiosis, common to both procedures, may be a factor in the activation of the miracidium. The additional, possibly synergistic, effect of light, suggests the presence of a threshold of necessary stimuli, any one if intense enough, or a combination, if less so, being able to initiate the process of eclosion.

SUMMARY

A method is described, with an illustrative experiment, whereby large numbers of miracidia of *Fascioloides magna* may be obtained when desired by exposure of embryonated eggs to cylinder nitrogen.

ACKNOWLEDGMENTS

The author is grateful to Dr. H. J. Griffiths of the College of Veterinary Medicine, University of Minnesota, for providing the eggs of *F. magna* used in this work. He is also indebted to Dr. N. R. Stoll of The Rockefeller Institute for his comments on the manuscript.

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STUDIES ON LARVAL *FASCIOLOIDES MAGNA*. IV. CHROMATOGRAPHIC ANALYSES OF FREE AMINO ACIDS IN THE HEMOLYMPH OF A HOST SNAIL

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Molluscs represent one of the largest animal phyla, but their physiology and biochemistry are poorly understood. Such work as has been done provides but slight acquaintance with their metabolism. In addition to interest attached to a study of this group per se, the habitual association of molluscs with trematode biology commends a special interest in them.

The biochemical relationships existing in intra-molluscan phases of fluke life cycles undoubtedly have much to tell the parasitologist. In order to define a part of the milieu in which these worms are found in one worm-host system, certain analytical studies of hemolymph of the snail *Lymnaea stagnalis jugularis* Say were undertaken and are here reported.

MATERIALS AND METHODS

Obtaining snail hemolymph. Hemolymph was secured from laboratory-reared *L. stagnalis jugularis* fed on lettuce or cereal (Pablum) as follows: Medium to large snails (at least 25 mm in shell length) were washed and placed on toweling to absorb excess moisture. A snail, with dry shell, was held spire down; then a 27-gauge needle on a 2-ml syringe was inserted (through a previously made shell perforation using a dissecting needle) into a sinus area adjoining the suture of the whorl and apical to the yellowish albumin gland (fig. 1).

Care was taken that the tip of the needle, its oblique opening preferably turned outward (and in view through the shell) did not puncture any internal organs or tear adjacent tissue. With gentle manipulation of the syringe, the opalescent hemolymph was cautiously withdrawn with the snail held as indicated in figure 1. This position has the advantage of allowing the hemolymph, aided by gravity, to drain downward to the point of removal. The procedure was terminated when the flow stopped and did not resume easily, as extended attempts at withdrawal of the fluid often resulted in contamination of the sample. Depending upon the size of the snail, 0.2 to 1.0 ml could be obtained. If not severely treated or excessive amounts of hemolymph removed, snails often recovered from the operation. Although done with care, this procedure was not performed using aseptic technique. Protein precipitant was added as soon as possible after withdrawal of hemolymph (or rarely, occasional samples for paper-chromatographic analyses were stored several days at 5 to 10 C prior to processing).

For paper chromatographic studies, samples

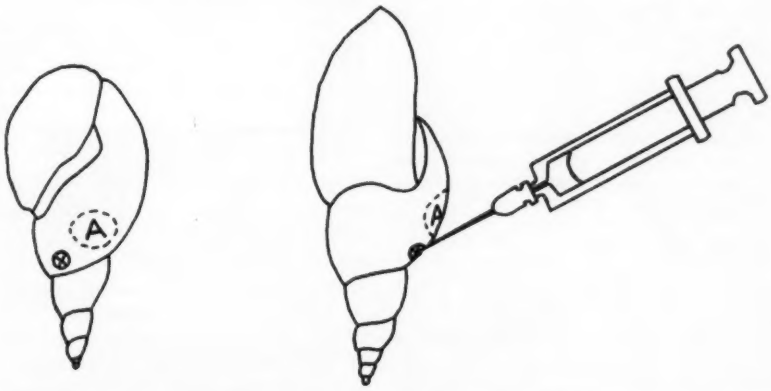

FIGURE 1. Point of insertion (X) of syringe for hemolymph withdrawal from *Lymnaea stagnalis jugularis*. The albumin gland, generally visible through the shell, is at A.

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were taken from snails recently removed from aquaria; for column chromatography, they were isolated without food for about 24 hours prior to the withdrawal of hemolymph. In all cases, analyses were performed on pooled samples, usually representing between 15 and 30 animals.

Preparation of samples. After centrifugation to remove cells, hemolymph for paper chromatography was diluted to 5 volumes with absolute ethanol. Precipitated protein was removed by centrifugation; the 80 percent ethanolic supernate was desalted (Plaisted, 1958). Collected eluates, evaporated to dryness, were taken up in an amount of 10 percent isopropanol giving, when possible, a 10-fold concentration over the original volume. The samples, stored at 5 to 10 C (generally to be used within several weeks and rarely after a few months) were applied to chromatograms in 50-microliter aliquots. Some samples were subjected to acid hydrolysis (6N HCl at 56 C for 2 to 4 days) to convert the amides, asparagine, and glutamine to their corresponding amino acids.

In preparation for column analysis, the centrifuged hemolymph was de-proteinized with picric acid (Stein and Moore, 1954). The pH was increased to 7.5 with NaOH for 4 hours at room temperature, followed by adjustment to pH 2.0 with HCl. This provided conditions favorable for the conversion of cysteine to cystine. Samples were refrigerated (usually at 5 to 10 C) until analysis. At most, this was about 6 weeks from the original withdrawal of hemolymph.

Chromatography. The one-dimensional paper method of McFarren (1951) was found useful for separation of the major amino acid constituents. Two solvent-buffer combinations were used: phenol-phosphate at pH 12.0, and m-cresol-borate at pH 8.4. Ascending chromatography was followed by washing in ether-acetone and spraying with the ninhydrin reagent of Moore and Stein (1954). Colors were allowed to develop at room temperature overnight.

In addition to the above analyses, the author was fortunate in being able to subject two samples prepared as previously stated to column chromatography (using Dowex 50) with the automatic recording apparatus described by Moore, Spackman and Stein (1958).

RESULTS

Figures 2, a and b show representative paper chromatographic patterns. In figure 2a, with phenol-phosphate buffer at pH 12, alanine, glycine, leucine (or isoleucine), and serine were readily detectable. Upon hydrolysis, aspartic and glutamic acids appeared, which indicated that asparagine and glutamine were present. Two unidentified spots were visible in the area between alanine and leucine. The pattern shown in figure 2b, with m-cresol-borate buffer at pH 8.4, wherein some substances of low Rf in figure 2a move even less, enabled the identification

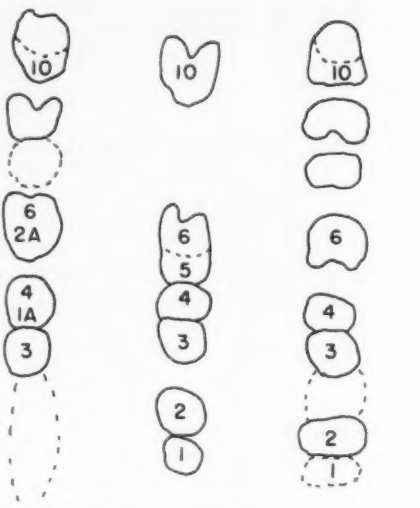

FIGURE 2a. Tracing of one-dimensional chromatogram of amino acids in hemolymph of *Lymnaea stagnalis jugularis* with phenol-phosphate buffer at pH 12 on Whatman No. 1 paper. (See also fig. 2b.) A, unhydrolysed hemolymph; B, hydrolysed hemolymph; S, S′, standards. Solid lines circumscribe distinct ninhydrin reactivity; broken lines surround areas where coloration was less clear or indicative of traces of reactant. The solid horizontal line indicates level of origin.

Key to amino acids: 1, aspartic acid; 1A, asparagine; 2, glutamic acid; 2A, glutamine; 3, serine; 4, glycine; 5, threonine; 6, alanine; 7, tyrosine; 8, valine; 9, methionine; 10, leucine*; 11, unknown.

* No distinction is made between isoleucine and leucine in these hemolymph samples.

of tyrosine and valine, and perhaps a trace of methionine. Slight unidentified ninhydrin activity here appeared in advance of the leucine position. Other analyses using isatin as a color reagent have given evidence of proline.

Figure 3 (in micromoles per 100 ml) and table I (in micrograms per ml) show results of column analyses of two separate samples. Sample A was equivalent to 4.0 ml of hemolymph, and sample B to 7.2 ml. Urea is seen to predominate as a ninhydrin reactive constituent. Other constituents appearing in one or both samples were: asparagine and glutamine (reported together), alanine α-aminobutyric acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and

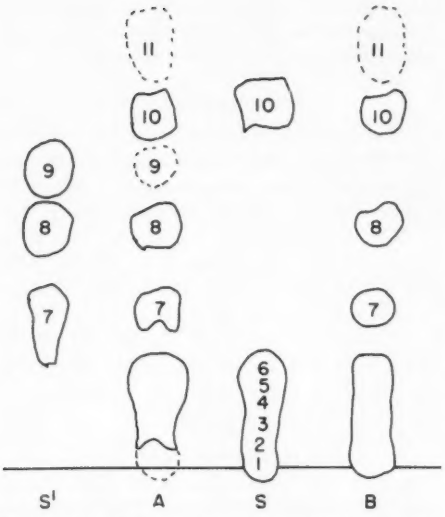

FIGURE 2b. Tracing of one-dimensional chromatogram of amino acids in hemolymph of *Lymnaea stagnalis jugularis* with m-cresol-borate buffer at pH 8.4 Whatman No. 1 paper. Abbreviations and key as shown in figure 2a.

valine. A trace of cystine appeared in one of the samples.

DISCUSSION

The qualitative results of the two types of analyses correspond well. Many of the amino acids present were demonstrated on paper chromatograms; the column method also detected α-aminobutric acid, glutamic acid, histidine, isoleucine and leucine (as separate entities), lysine, ornithine, phenylalanine, threonine, tryptophan, a trace of cystine, and urea. Quantitatively, the latter analysis should be accurate to about 10 percent at the concentrations present in the sample. Although accidental preparative losses will have slightly lowered the total amino acid content determined, their relative concentrations are believed to be within the above limits.

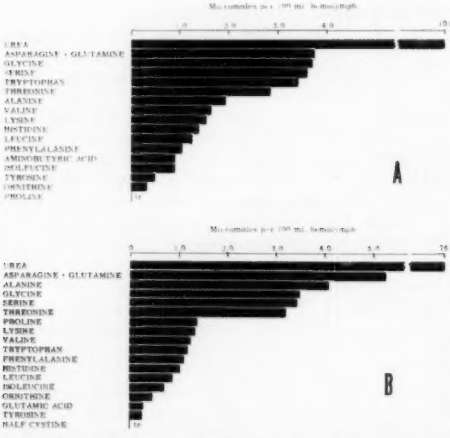

FIGURE 3. Amino acid constitution of two hemolymph samples from *Lymnaea stagnalis jugularis* as determined by column chromatography. (Trace amounts are designated by tr.)

TABLE I. *Results of two hemolymph samples from* Lymnaea stagnalis jugularis *analysed by automatic recording column chromatography using Dowex 50.* (See text). *Values given are derived from data of figures 3, A and B.*

	MICROGRAMS PER ML	
	A	B
α-Alanine	1.7	3.6
α-Aminobutyric Acid	0.9	—
Asparagine and Glutamine*	5.5	7.7
Cystine	—	trace
Glutamic Acid	—	0.4
Glycine	2.8	2.6
Histidine	2.2	1.6
Isoleucine	1.2	0.9
Leucine	1.6	1.2
Lysine	2.3	1.9
Ornithine	0.4	0.6
Phenylalanine	1.7	1.8
Proline	trace	1.6
Serine	3.8	3.6
Threonine	3.4	3.8
Tyrosine	0.9	0.5
Tryptophan	7.0	2.4
Valine	1.9	1.5
Urea	61.0	46.0

* Reported together with weight calculated as glutamine.

The relatively dilute solution of amino acids in *Lymnaea* hemolymph may be responsible for the inability to detect others one might expect in such samples, e.g., arginine and aspartic acid. In view of the absence of glutamic acid in sample A (fig. 3), it is possible that its presence (a relatively small amount) in sample B resulted from the hydrolysis of glutamine during preparation of the sample. β-alanine, reported from certain invertebrates (Simpson et al, 1959) was not found, nor was there evidence of taurine, its absence being in accordance with the data of Simpson, Allen, and Awapara (1959), who did not detect it in other freshwater molluses (as opposed to its presence in marine forms). In such comparisons, however, distinction should be made between free amino acids in extracts of whole organisms (referred to above) and those found in hemolymph analyses such as are reported for *Lymnaea* in this paper.

Fasting snails were deliberately chosen as the source of hemolymph studied by column chromatography in order to provide a baseline for any later determinations on snails fed various types of food. No efforts were made in this study to correlate quality or quantity of food available to the snails (lettuce or Pablum cereal) with the amino acid content of their hemolymph.

This is believed to be the first reasonably complete analysis of free amino acids in the hemolymph of *Lymnaea stagnalis jugularis*. Say. It was undertaken to correlate certain single amino acids found to extend the survival of larval *Fascioloides magna* (Bassi, 1875) Ward, 1917, in vitro (Friedl, 1960a, b) with those found in the snail hosts hemolymph. Of the three amino acids seen to favor survival most noticeably in previous experiments (hydroxy-proline, proline, and serine), only serine appears to be present as a major constituent in the samples from fasting snails. Generally speaking, however, alanine, glycine, serine, and threonine, figuring prominently in the analyses shown in figure 3, all have given some evidence of extending longevity in vitro. The amide, asparagine, also favoring survival and possibly serving as a source for aspartate, (which in an unpublished experiment likewise appeared effective in prolonging survival) may also be considered in this respect.

SUMMARY

Free amino acids in hemolymph of a laboratory-reared snail, *Lymnaea stagnalis jugularis* Say, have been studied by means of paper and column chromatography. Ninhydrin positive substances detected include the following: Urea, asparagine and glutamine, α-alanine, α-aminobutyric acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and traces of cystine and perhaps methionine.

ACKNOWLEDGMENTS

The author is indebted to Dr. S. Moore of The Rockefeller Institute for his interest and advice concerning determinations using automatic-recording column chromatography, and to Miss Anna Zamoyska for her technical assistance in these determinations. He is grateful to Dr. N. R. Stoll, also of the Rockefeller Institute, for his comments on the manuscript.

Information regarding the identity of the snails used in this study was most kindly given by Drs. H. A. Rehder and J. P. E. Morrison of The U. S. National Museum.

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LIFE CYCLE OF *ACANTHOPARYPHIUM SPINULOSUM* JOHNSTON, 1917 (ECHINOSTOMATIDAE: TREMATODA)

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The genus *Acanthoparyphium* was established by Dietz (1909) with *Echinostomum phoenicopteri* Lühe, 1898 as type species. *A. phoenicopteri* was recovered from the intestine of *Phoenicopterus roseus* captured in Tunis. Johnston (1917) described a new species, *A. spinulosum*, from the duodenum of a golden plover, *Charadrius dominicus*, captured near Sydney, Australia. Yamaguti (1934) found large numbers of *A. spinulosum* in the small intestine of *Squatarola squatarola* from Kanagawa Prefecture, Japan. In this same paper, he described what he believed to be the redia, cercaria and metacercaria of an *Acanthoparyphium* from a snail, *Batillaria multiformis* (Lischke), collected at Takasago, Hyôgo Prefecture; however, no experimental work was done. Homer T. Russell, Jr. (personal communication) has found *A. spinulosum* in the intestines of the black-bellied plover, *Squatarola squatarola*, and the avocet, *Recurvirostra americana*, taken at Morro Bay, California. Skrjabin (1956) reviewed the genus *Acanthoparyphium* and listed eight species. Yamaguti (1958) lists a total of 10 species (one a subspecies) from the intestines of birds taken in Australia, Japan, Korea, the Philippines, or Tunis.

An abstract of the present work has been published (Martin and Adams, 1960).

MATERIALS AND METHODS

Naturally infected, brackish-water snails were collected in Upper Newport Bay, California. These were isolated in finger bowls where they gave off large numbers of cerariae. Uninfected snails were exposed to cercariae which penetrated and encysted. Crushed snails furnished stages earlier than the cercarial. Snail tissue containing metacercariae was fed on several occasions to hatchery-raised chicks, preferably before the chicks had started feeding. The chicks were autopsied and adult worms were recovered from their small intestines. Eggs obtained from adult worms were hatched on several occasions and the miracidia were studied alive or fixed and stained. Some miracidia were observed alive under oil immersion or were stained with silver nitrate to determine the number of epidermal plates. Living cercariae were studied under oil immersion to work out the details of the excretory system. Whole mounts were fixed in 10% formalin or Heidenhain's fixative; stained with silver nitrate, Mayer's paracarmine or Celestin blue B; and mounted in Permount.

All measurements are expressed in millimeters.

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OBSERVATIONS AND DISCUSSION

Miracidium (Fig. 1)

Eggs were obtained from experimental infections by teasing apart the adult worms. The daily observation of miracidial development indicated that eyespots became evident after 8 or 9 days. Miracidia were moving within the egg shells by the 15th day. Hatching time varied from 16 to 27 days. In one batch of 153 eggs incubated for 21 days, 48 had hatched, 73 contained eyespotted miracidia and 32 were delayed in development. At the end of 27 days all but 10 had hatched and these contained dead miracidia or disintegrating blastomeres. The hatched miracidia usually swam forward with the eyespot up and with about a 90° roll of the body from side to side. Sometimes the direction of swimming was a straight line but sudden turns were common. If some obstruction impeded their progress the miracidia would bend their bodies. A few calculations were made of the rate of swimming which averaged about 1.28 mm per second.

Measurements of 20 miracidia are: body length 0.078 to 0.111, average 0.104; maximum width 0.028 to 0.039, av. 0.034; eyespot av. diam. 0.01. The body is clothed with long cilia which are born by four tiers of epidermal plates. The latter number, from anterior to posterior, 6–8–4–2. Other studies on the number of echinostome miracidial epidermal plate cells (Mathias, 1925; Rasín, 1933; Beaver, 1936; Nasir, 1960) list 6–6–4–2. However, it is difficult to see how six cells could complete the second tier when five cells can be seen from one side of the miracidium of *Echinostoma nudicaudatum* in figure 2 plate

I of Nasir's (1960) paper. The epidermal plate cell numbers were consistent for the six miracidia of *A. spinulosum* studied under oil immersion. Lynch (1933) found some variation of epidermal plate numbers in one hundred miracidia of *Heronimus chelydrae* MacCallum and perhaps some variation might be found in a similar number of *A. spinulosum* miracidia. The miracidium of *A. spinulosum* has glands and a large black eyespot in the anterior part of the body and a median mass of germinal cells and a pair of flame cells more posteriorly.

Rediae (Figs. 5, 6)

Infections of *A. spinulosum* are not common in *Cerithidea hegewischi californica* Haldeman; only six infections were found in 12,995 snails examined by crushing (Martin, 1955). Two generations of rediae develop in the digestive glands of the snail host. The redial body wall contains orange-red spots. This pigment was shown by Nadakal (1960) to be carotene obtained unaltered from the snail host and in turn taken by the snail from its algal food. Redial size varies a great deal with age but the size of the pharynx is nearly as great in small as it is in large rediae. Small rediae have two lateral lappets near the posterior end and a collar near the anterior end. The gut in small rediae extends most of the body length. In larger rediae the lateral lappets disappear but the collar is still present. Measurements of 20 larger rediae are: body length 0.379 to .91 av. 0.51; maximum body width 0.168 to .252, av. 0.203. The nearly spherical pharynx is 0.034 to .0434 long and wide, with slightly greater average length, 0.0393, than width, 0.0391. Germ balls and maturing cercariae were present in these rediae as well as a birth pore which opens near the anterior end of the body.

Cercaria (Figs. 3, 4)

The body surface is smooth except for scattered papillae, each with a short bristle, and a single row of 23 oral spines, which is interrupted ventrally. The oral sucker is subterminal and is smaller than the approximately medially located ventral sucker. Several types of glands occur; two with irregular margins are dorsal to the oral sucker. Their ducts open near the anterior margin of the oral sucker. Four glands with cell bodies between the pharynx and the ventral sucker have ducts which pass anteriorly to separate and open near the anterior margin of the oral sucker. Scattered glandular masses of short rods and granules extend from a little posterior to the pharynx to near the posterior end of the body. These are cystogenous glands which are exhausted in forming the metacercarial cyst wall. Several masses of deeply staining cells are located near the acetabulum, one near its anterior margin and two posterior to it, that are primordia of the reproductive system. The mouth leads almost immediately into a spherical pharynx that connects to a fairly long esophagus. Intestinal bifurcation occurs just in front of the acetabulum and the ceca extend laterally and posteriorly to near the posterior end of the body.

The excretory system is rather complex. The excretory bladder is cylindrical with a thickened and indented wall. An exit duct passes from the bladder into the tail then bifurcates to lateral openings. A thin-walled main collecting vessel proceeds anteriorly on each side of the body to about the mid-acetabular level where branching begins and is continued to the pharyngeal level. The main collecting vessels contain numerous concretions, some of which adhere to the vessel walls. From the mid-anterior region of the branched vessel a duct proceeds anteriad to loop back upon itself at the mouth level and proceeds posteriad to the post-acetabular level where it divides into two ducts, one of which supplies the anterior body quadrant and the other the posterior. Each of these ducts divides into two more and each of these bifurcates to receive two pairs of flame cells by each branch. Thus there are 32 flame cells arranged in pairs on each side of the body. Tufts of cilia occur in the ascending duct that joins the branched portion of the excretory system. The excretory system was difficult to work out because of the glands, concretions, and some pigment.

The tail is simple with many nuclei and is whipped in a figure-eight pattern during swimming. After swimming for a time, the cercariae may be taken into the mouth of *Cerithidea h. californica* where they penetrate and encyst. Measurements of 20 cercariae fixed in cold Bouin's solution, stained and mounted are: body length 0.255 to .398, av. 0.317; maximum body width 0.124 to .177, av. 0.156; oral sucker diam. 0.037 to .047, av. 0.042; acetabular length 0.044 to .065, av. 0.052; acetabular width 0.053 to .072, av. 0.059, pharynx length 0.015 to .022, av.

0.019; pharynx width 0.016 to .019, av. 0.016; esophagus variable in length, approximately 0.08; tail length 0.37 to .435, av. 0.415; maximum tail width 0.025 to .028, av 0.026.

Metacercaria (Figs. 7, 8)

The metacercariae encyst in the buccal mass of the snail, in radular muscle, or connective tissue. About 90 percent of the larger *Cerithidea h. californica* were naturally infected with various numbers of metacercariae, usually not more than 6 per snail although much heavier infections were experimentally induced, sometimes between 60 and 70. The metacercariae grow to several times the cercarial size. The 23 oral spines become much more conspicuous than in the cercaria. They are 0.017 to .024, av. 0.021 long and 0.0039 to .005, av. 0.004 wide. The second and third spines from the ventral ends of the row are a little larger than the rest. Small spines develop on the surface of the anterior half of the body. The oral sucker glands are still quite evident, but the other cercarial glands are less conspicuous or, in the case of the cystogenous, have disappeared. The digestive tract is similar to that of the cercaria. The larger vessels of the excretory system are filled with concretions. The genital ducts are partially developed. Radular masses containing metacercariae were removed from the snails and fed to hatchery-raised chicks.

Adult (Figs. 9, 10)

Adult worms were removed from the second half of the duodenum and anterior portion of jejunum of the chick hosts. The worms start egg production when about 12 days old. They continue to grow until they are about 1 month old. Oral and body surface spines increase in size. The latter are scale-like and cover the dorsal body surface down to the genital pore, but laterally and especially ventrally they extend to the level of the posterior testis. The posterior end of the body frequently is bent ventrally and bears a small "tail like" protuberance. Oral glands of the cercaria are still present. The oral sucker is much smaller than the acetabulum. The digestive tract is essentially the same as in the metacercaria. The stem of the excretory bladder is elongated and reaches to near the hind margin of the posterior testis. The posterior end of the bladder has a thick, folded, apparently non-cellular lining that is surrounded by a rosette of glands. Possibly these glands secrete the lining. The excretory pore is subterminal. The rest of the major excretory ducts are like those of the metacercaria. Small excretory concretions can still be found in 14-day-old worms. The anterior testis is usually wider and shorter than the posterior. Ducts proceed anteriorly from the testes, unite, then penetrate the cirrus sac. The cirrus sac is elongate, extending posterior to the acetabulum almost to the ovarian level. It contains a seminal vesicle, some prostate cells and a long cirrus which can be extruded from the body at some length (fig. 9). The cirrus bears some spines with large basal plates that are best seen in living specimens. The spherical to oval ovary is located in the posterior part of the first half of the body, usually a little to the right of the median sagittal plane. An oviduct leads off the posterior surface of the ovary and before entering the ootype is joined by Laurer's canal, which opens dorsally. The main vitelline ducts also join the oviduct. The vitelline follicles extend from the ovarian level to near the ends of the ceca. They are mainly lateral to the ceca but may overlap them especially posterior to the posterior testis. The uterus extends from the ootype to the genital pore, making loops on each side of the body between the ootype and the acetabulum. The ootype is located between the ovary and the anterior testis and is surrounded by gland cells. The genital pore is immediately posterior to the gut bifurcation at or near the mid-ventral surface of the body. The eggs are yellow, oval, and operculated. They number 16 to 35 in 17-day-old worms, but in month-old specimens they may number over 150.

Measurements of 20 specimens from 17 to 30 days of age are: body length 3.32 to 5.53, av. 4.09; maximum body width 0.6 to 1.22, av. 0.83; cuticle 0.006 to .009, thicker in anterior part of body; collar spines 0.05 to .072, av. 0.063 long and 0.012 to .022, av. 0.017 wide; body spines 0.009 to .016, av. 0.014 long and 0.006 to .011, av. 0.009 wide, smaller in neck region; oral sucker length 0.096 to .149, av. 0.126; oral sucker width 0.103 to .165, av. 0.127; acetabular length 0.342 to .473, av. 0.388; acetabular width 0.351 to .535, av. 0.413; prepharynx 0 to .09 long; pharynx 0.093 to .137, av. 0.113 long and 0.059 to .131, av. 0.092 wide; esophagus 0.373 to .70, av. 0.493 long and 0.019 to .059, av. 0.043 wide; ovary 0.115 to .230, av. 0.164 long and 0.109 to .205,

av. 0.153 wide; eggs 0.084 to 0.106, av. 0.096 long and 0.056 to .068, av. 0.064 wide; anterior testis 0.34 to .644, av. 0.41 long and 0.24 to .532, av. 0.36 wide; posterior testis 0.406 to .756, av. 0.535 long and 0.196 to .560, av. 0.294 wide; cirrus sac 0.588 to 1.134, av. 0.825 long and 0.07 to .196, av. 0.127 wide.

To the best of our knowledge, this is the first experimental demonstration of a life cycle in the genus *Acanthoparyphium*. The genus probably has a world-wide distribution.

SUMMARY

The life cycle of *Acanthoparyphium spinulosum* has been traced experimentally. The first and second intermediate host is the brackish-water snail, *Cerithidea hegewischi californica*, collected at Newport Bay, California. The complete excretory system has been traced in the cercaria, and it includes 32 flame cells in pairs on each side of the body. Metacercariae encyst only in the radular muscle and adjacent tissues of the snail. Adult worms were recovered from the lower duodenum and upper jejunum of experimentally infected hatchery-raised chicks. Egg production begins when the adults are about 12 days old. Adults increase in size after starting egg production.

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EXPLANATION OF PLATES

All figures concern *Acanthoparyphium spinulosum*.

Drawings made with aid of camera lucida unless otherwise stated.

Abbreviations used: A, acetabulum; C, cirrus sac; CG, cystogenous gland; Ci, cirrus; CO, excretory concretion; E, excretory bladder; ES, eyespot; F, flame cell; G, genital pore; GC, germinal cells; Gl, gland cells; GP, genital primordium; GU, gut; L, lateral appendage; M, metacercaria; O, oral sucker; OG, oral gland; OO, ootype; OV, ovary; P, pharynx; R, radula; T, testes; V, vitellaria.

FIGURE 1. Miracidium.
FIGURE 2. Egg with operculum raised on one side.
FIGURE 3. Cercaria.
FIGURE 4. Diagram of cercarial excretory system.
FIGURE 5. Redia.
FIGURE 6. Young redia.
FIGURE 7. Radular mass with metacercariae.
FIGURE 8. Metacercaria.
FIGURE 9. Extruded cirrus.
FIGURE 10. Adult.

PLATE I

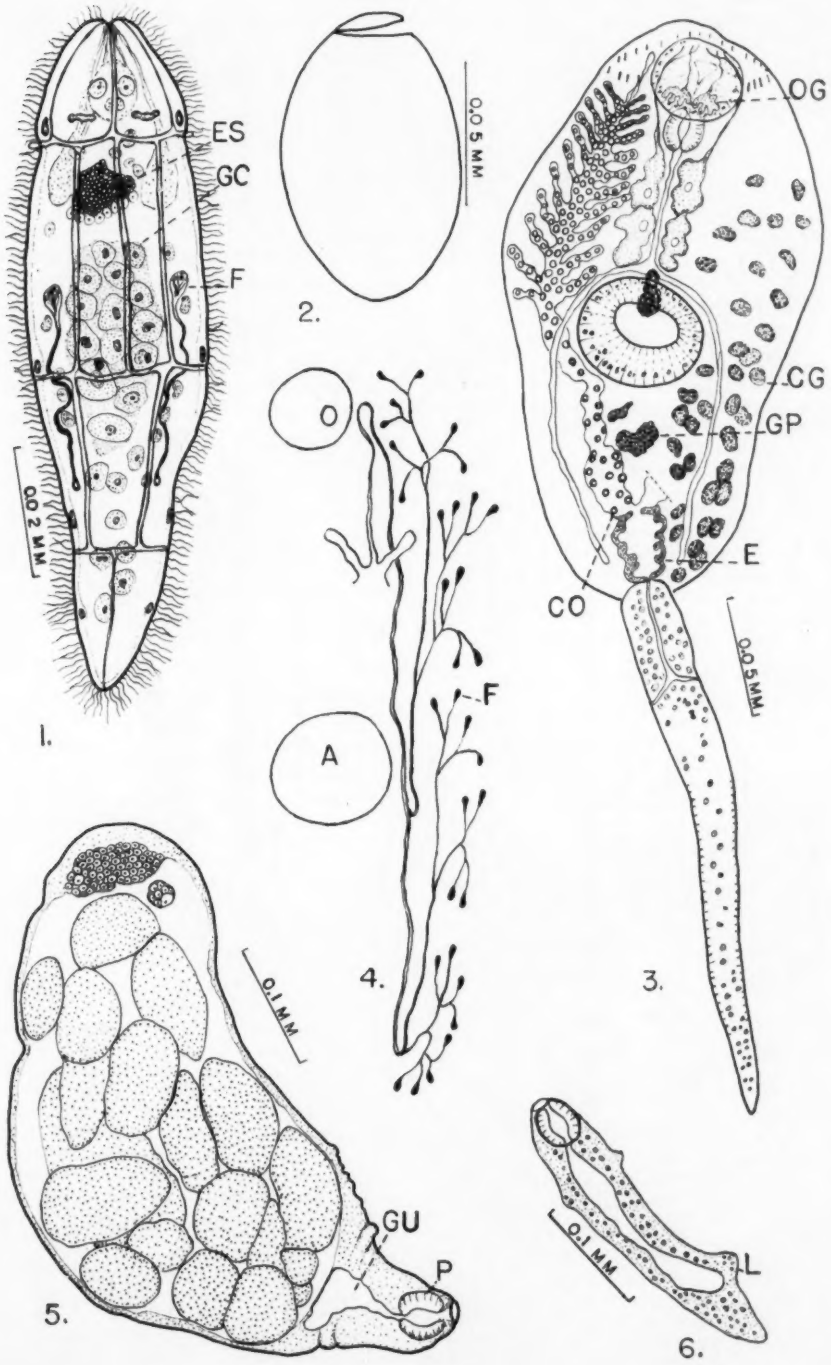
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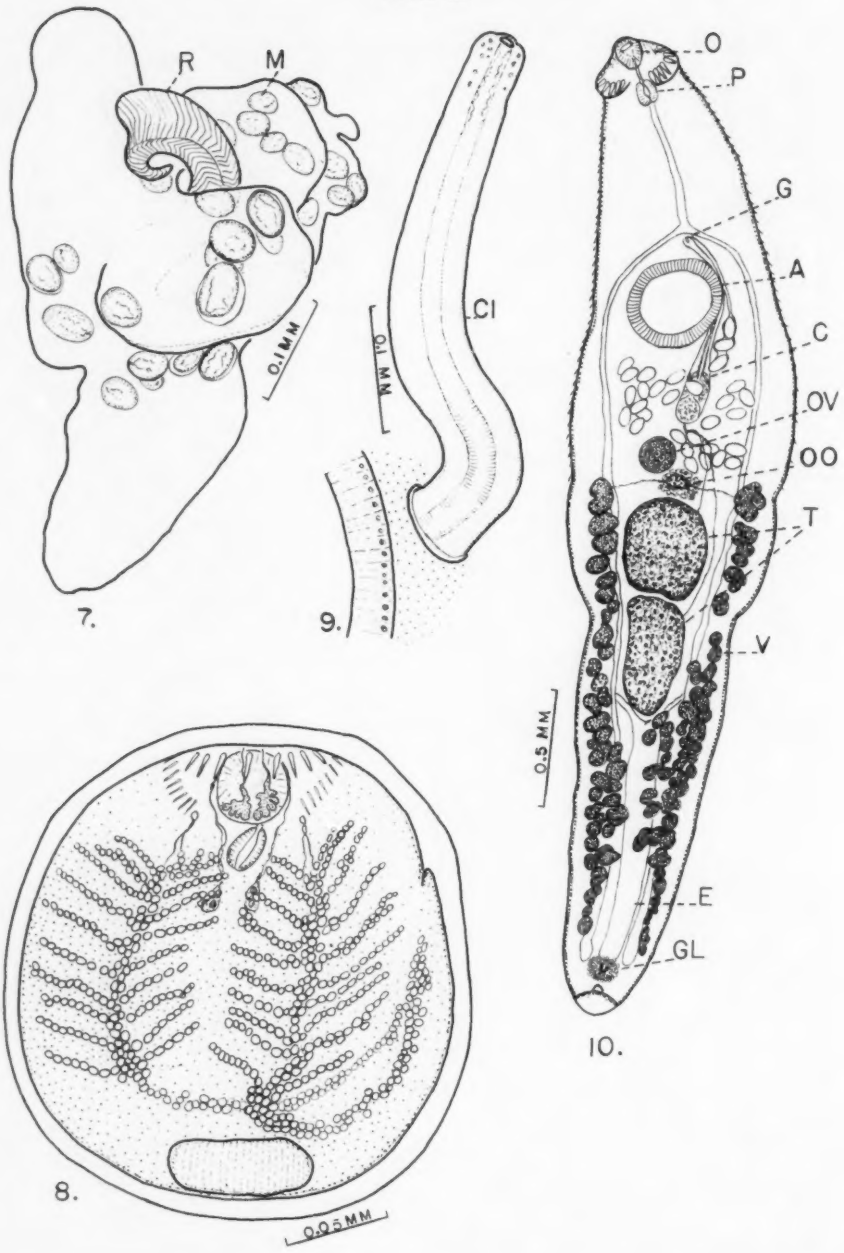
PLATE II



GRYSOMA SINGULARIS, A NEW SPECIES OF TREMATODES (DIGENEA: PSILOSTOMIDAE) FROM THE RACOON *PROCYON LOTOR* (L.)

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During October 1959, one of us (B.J.B.) recovered 28 specimens of an undescribed trematode from the small intestine of a racoon, *Procyon lotor* (L.). The host animal had been taken from Bulloch County, Georgia. All of the specimens were stained and mounted in their entirety. After all specimens had been studied for general morphological features, three of them were dismounted and sectioned serially. It was concluded that the organism represented a new genus and species having features sufficiently distinctive to warrant the erection of a new subfamily for it. The new subfamily is assigned to the family Psilostomidae Odhner, 1913.

The subfamily and generic names designated, *Grysominae* and *Grysoma*, respectively, refer to the very small body of the parasite while the species name, *singularis*, indicates that no more than a single egg was observed in the uterus of any specimen.

All measurements are given in millimeters and all specimens available were measured. In the text measurements are given as mean followed in parentheses by the range.

Grysominae subf. n.

Diagnosis: Psilostomidae. Small to medium-sized distomate trematodes having pyriform, spinose body and subequal suckers. Prepharynx, pharynx and esophagus present. Ceca extending to near caudal end of body. Testes rounded to triangular in shape, diagonally situated in body behind level of acetabulum. Cirrus pouch small, containing seminal vesicle, cirrus and prostatic gland cells. Genital pore ventral, median, between bifurcation and acetabulum. Ovary to one side of midline, in region of testes or close behind acetabulum. Uterus short, containing from none to very few ova. Vitellaria composed of many large cells which may be grouped into strongly or weakly defined follicles, lateral and dorsal in position, usually in area of ceca. Excretory pore ventral. Excretory bladder with median stem to level of testes where it divides into two arms which extend to level of pharynx. Parasitic in intestine of birds and mammals.

Type genus: Grysoma gen. n.

Grysoma gen. n.

Diagnosis: Grysominae. Body small, pyriform, relatively thick, with cuticular spines to level of testes. Oral sucker and acetabulum well developed: oral sucker subterminal, smaller than acetabulum: acetabulum in anterior half of body. Prepharynx present. Pharynx well developed, entire. Esophagus short. Ceca reaching to near posterior end of body. Testes semi-lunar to triangular in shape, diagonally situated in area midway between acetabulum and posterior end of body. Vasa efferentia uniting after entering cirrus pouch. Cirrus pouch moderately well developed, sometimes reaching as far back as caudal boundary of acetabulum, containing seminal vesicle, cirrus and prostatic gland cells. Genital pore median, ventral, between intestinal bifurcation and acetabulum, usually with strong posterior lip. Ovary round to transversely oval, usually close behind acetabulum, slightly to right of midline. Mehlis' gland small. Laurer's canal and seminal receptacle absent. Uterus short, proximal end with sperm cells. Egg large, no more than one in a uterus. Vitellaria lateral and dorsal to ceca, extending from level of genital pore to about midway between ends of ceca and caudal end of body, follicles poorly defined, each consisting of a few large cells. Yolk reservoir very large. Excretory pore ventral, near posterior end, guarded by sphincter muscle. Excretory bladder with median stem dividing into two prominent arms at level of testes; arms extending to level of pharynx, with many diverticula. Parasitic in intestine of mammals.

Type species: G. singularis sp. n.

Grysoma singularis gen. n. and sp. n.
(Plate I, figs. 1–5)

Description: Body pyriform in shape (figs. 1 and 2), more pointed anteriorly, about half as thick as broad (fig. 5), 0.42 (0.29 to 0.48) long by 0.18 (0.07 to 0.26) wide; widest behind level of acetabulum. Cuticle moderately thick, beset with short, strong spines; spines arranged in spiraled rows, rows incomplete anterior to acetabulum, leaving broad midventral strip of cuticle and cap-like anterior extremity free of spines; spines stronger and more numerous in anterior region, disappearing mid-ventrally and mid-dorsally a short distance anteriorly to their termination laterally, terminating completely at level of testes; first two or three rows of spines on ventral surface behind acetabulum stronger than spines in adja-

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cent rows and often pulled close to caudal lip of acetabulum (especially when sucker is sunken into underlying tissues) giving impression of specially arranged spines in area. Oral sucker subterminal, with cavity and opening directed forward (fig. 3), 0.05 (0.04 to 0.06) long by 0.05 (0.03 to 0.06) wide. Prepharynx moderately long, often allowing pharynx to lie completely exposed behind oral sucker. Pharynx muscular, longer than broad, 0.04 (0.03 to 0.05) long by 0.03 (0.02 to 0.04) wide. Esophagus short, about 0.02 long, bifurcating short distance in front of acetabulum. Ceca of about uniform diameter, diverging slightly in passing laterally to acetabulum, curving inward slightly at posterior limits, ending midway between testes and caudal end of body, lined internally by rather flat to pyramidal-shaped epithelial cells. Acetabulum with posterior border near middle of long axis of body, larger than oral sucker, 0.08 (0.05 to 0.10) in diameter.

Testes diagonal in position, half-moon to triangular in shape, pressed close together, about midway between acetabulum and caudal end of body (in one specimen the anterior testis lay partially hidden by the ventral sucker while in another specimen (fig. 1) four testes are present); anterior testis located from posterior border of acetabulum to 0.07 behind that organ (more often the greater distance), 0.07 (0.04 to 0.10) in greatest diameter and 0.05 (0.03 to 0.07) in cross section; posterior testis overlapping area of anterior testis by about half its length, 0.08 (0.05 to 0.12) long by 0.06 (0.03 to 0.09) wide. Vasa efferentia uniting after entering cirrus pouch. Vas deferens expands immediately into small but distinct seminal vesicle. Cirrus short, muscular. Cirrus pouch slightly J-shaped, extending directly dorsad short way from pore before turning posteriorad to end in front of acetabulum or somewhere between this point and caudal boundary of acetabulum; many rather large prostatic gland cells in cirrus pouch (fig. 4). Genital pore ventral, median, between intestinal bifurcation and acetabulum (sometimes the genital pore appears as a cavernous opening guarded by a prominent fold of muscle and cuticle posteriorly and separating it from anterior margin of acetabulum (figs. 3 and 4), and having male organ opening through lateral wall of cavern into the genital atrium).

Ovary close behind acetabulum or lying partly covered by latter, round to ovoid in shape, 0.04 to 0.05 in size, lying very slightly to right of midline. Oviduct short. Ootype surrounded by cells of Mehlis' gland. Seminal receptaculum and Laurer's canal absent. Uterus short, leading directly forward dorsal to acetabulum, to cavernous genital atrium, containing no more than single egg (only 7 of 28 specimens contained an egg in uterus). Egg thick shelled, 0.10 (0.08 to 0.12) long by 0.06 (0.05 to 0.10) wide. Vitellaria in lateral fields and dorsal to ceca, beginning at about level of genital pore and terminating beyond ends of ceca (usually midway between ends of ceca and caudal end of body although in one specimen (fig. 2) vitellarium on one side of body ends before reaching end of cecum on that side while in another specimen a definite break occurred in vitellarium on one side in region of testes), composed of numerous large vitelline cells, each cell with clearly defined nucleus and numerous cytoplasmic granules; cells organized into weakly defined follicles. Transverse yolk ducts delicate, uniting to form large yolk reservoir. Reservoir occupying most of area between testes and acetabulum; yolk cells in reservoir about same size as cells in vitellaria but differing from them in being much paler, less distinctly outlined, with fewer cytoplasmic granules and in having less well defined nuclei.

Excretory pore ventral, near posterior extremity, guarded by muscular sphincter. Excretory bladder thin-walled, passing inward and forward from pore to form narrow, deep vesicle, bifurcating at caudal boundary of testes into two narrow but deep arms (figs. 1, 2, 4 and 5); arms ultimately becoming one-half to two-thirds as deep as thickness of body (fig. 5), passing forward to level of pharynx and with increasing numbers of lateral (both sides of each arm) diverticula, (more clearly seen in sectioned material (figs. 4 and 5); diverticula spreading beneath acetabulum to such an extent as to produce a cistern-like space of sufficient size for that organ to sink into during certain movements of the body.

Host: *Procyon lotor* (L.).

Habitat: Small intestine.

Locality: Statesboro (Bulloch County), Georgia.

Type and additional specimen: U. S. Natl. Museum Helminthological Collection. Slide No. 39093.

Grysoma singularis is currently assigned to the family Psilostomidae Odhner, 1913. The relationship of the family to the families Echinostomatidae Poche, 1926, and Cathaemasiidae Fuhrmann, 1928, has been the subject of considerable discussion among previous workers. Suffice it to say here that the three families have many anatomical features in common and often it becomes difficult to conclude that any material at hand belongs to one and not to another of the three.

Among the flukes currently assigned to the family Psilostomidae the newly described form stands alone in these important respects: (1) the testes are trinagular (or half-moon) in shape; (2) the testes are diagonally arranged in the middle portion of the body behind the acetabulum; (3) the zones of the testes strongly overlap, leaving the organs pressed closely together; (4) the very short uterus was not seen to contain more than a single egg; (5) the egg is approximately one-fourth as large as the entire body; (6) the vitellaria is distinctly cellular; and (7) the yolk reservoir is very large. These features appear to be of sufficient strength and of such constant appearance (28 specimens) not only to separate the form from the remainder of the family as a new genus, but to

justify the erection of a new subfamily for its reception.

The new subfamily bears a close resemblance to the subfamily Psilostominae Lühe, 1909, in the general arrangement of the internal organs. The diagonally arranged testes, the triangular shaped testes, the short uterus and the very large, single egg "separating the testes from the acetabulum" readily distinguishes the new subfamily from the Psilostominae forms. The diagonal arrangement of the testes in the new subfamily suggests a possible relationship with the only known example of the subfamily Sphaeridiotrematinae Yamaguti, 1958. Here however, the diagonal arrangement of the testes appears to be more a dorso-ventral overlapping rather than a lateral one as in *Grysoma*. Also, in the subfamily Sphaeridiotrematinae the testes are located very near the caudal extremity of the body, and the ovary is immediately lateral to the anterior testis. The position of the genital pore, the size of the acetabulum, the extent of the vitellaria and the number of eggs in the "winding" uterus in the Sphaeridiotrematinae are of further assistance in separating this subfamily from Grysominae. From the subfamily Apopharynginae Yamaguti, 1958, the new subfamily differs in its possession of a well developed, muscular pharynx while the absence of a peduncle in association with the ventral sucker immediately distinguishes the new subfamily from the subfamily Stephanoproraoidinae Yamaguti, 1958.

Among the forms currently assigned to the family Psilostomidae a single species, *Psilostomum marilae* Price, 1942, closely resembles *G. singularis*. This species, *P. marilae*, was recovered along with *Sphaeridotrema globulus* (Rud. 1814) from the lesser scaup duck, *Marila affinis* (Eyton), in Maryland. Dr. Price compared his new species with *Psilostomum progeneticum* Wisniewski, 1933, a species which was described from precocious (neotenic) metacercariae from *Rivulogammarus spinicaudatus* and *Fontogammarus bosniacus* in Poland and which was presumed to reach sexual maturity in aquatic birds. A careful check of the characters displayed by *Psilostomum progeneticum* suggests it does not belong to the Psilostomidae, and that when more information on the species is available the form may well be assigned to the family Allocreadiidae.

Through the kindness of Mr. Allen McIntosh, U. S. Department of Agriculture, Bureau of Animal Industry, Beltsville, Maryland, the authors have been privileged to examine eight specimens of *Psilostomum marilae*. These specimens confirm Mr. McIntosh's opinion that *G. singularis* and *P. marilae* are congeneric. Both organisms conform to the diagnosis of our new genus, *Grysoma*, rather than to the genus *Psilostomum* Looss, 1899, and hence *P. marilae* is transferred to *Grysoma* as an additional species. The name becomes *Grysoma marilae* (Price, 1942).

Grysoma singularis differs from *G. marilae* in that the body is smaller, the vitellaria is grouped into less distinct follicles and is less extensive, the ventral sucker is smaller in relation to the size of the oral sucker, the ovary is distinctly more anterior (nearer the ventral sucker rather than at the level of the anterior testes) and the egg is larger. It should be pointed out, however, the differences indicated for these two organisms may be due to the fact that they come from different host species, *G. marilae* from ducks and *G. singularis* from the raccoon. When the life cycle is known for each of them, it may become necessary to place *G. singularis* in synonymy with *G. marilae*.

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EXPLANATION OF PLATE I *

Grysoma singularis

Figure 1. Whole mount. Dorsal view. Note size of yolk reservoir and presence of four testes.

Figure 2. Whole mount. Ventral view. Note size of egg in uterus and the uneven distribution of vitelline "follicles."

PLATE I

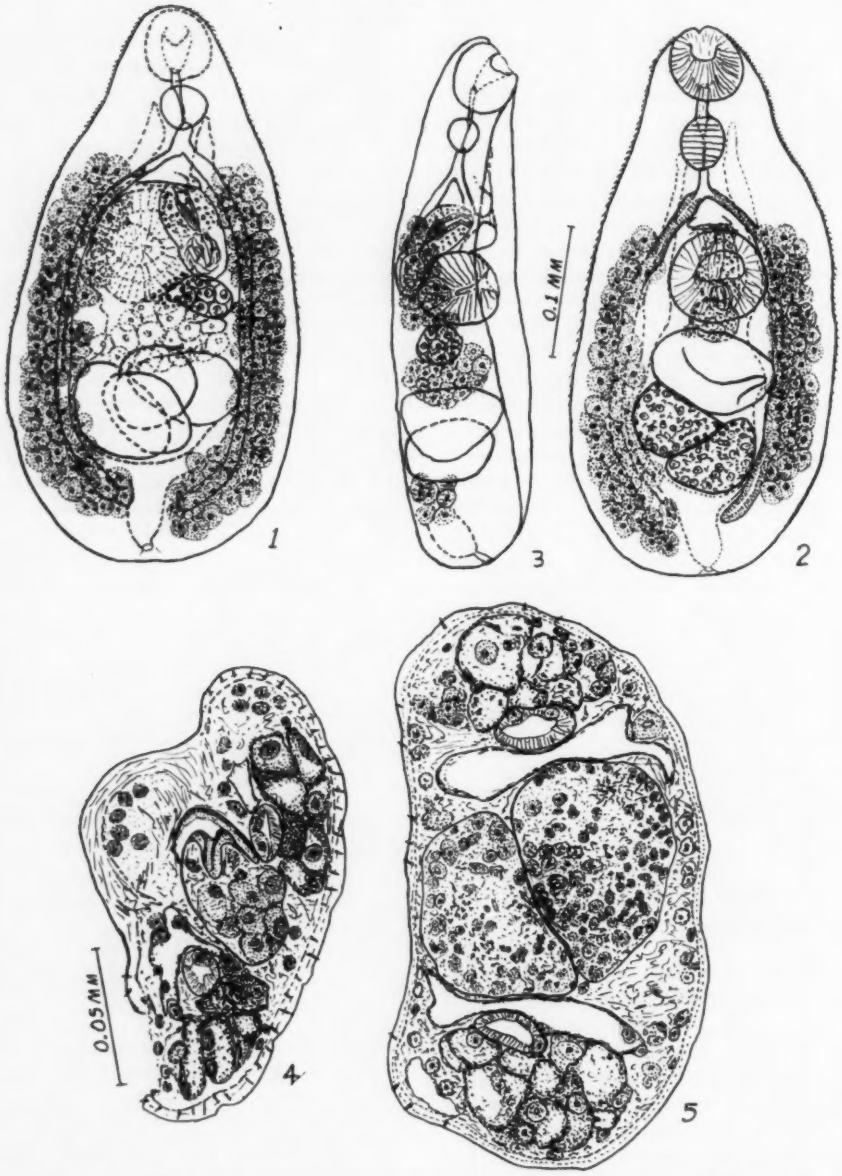

Figure 3. Whole mount. Profile view to show prominence of posterior lip of genital pore, size of yolk reservoir and retracted condition of the ventral sucker. Note location of excretory pore and bladder. Vitellaria are shown at anterior and posterior limits only.

Figure 4. Cross section through posterior lip of the genital pore, the genital atrium and cirrus pouch. Note large prostatic gland cells in cirrus pouch and the arrangement of the vitelline "follicles" about the ceca.

Figure 5. Cross section through testes. Note the depth of the arms of the excretory bladder and the presence of diverticula.

* All figures were made with the aid of the camera lucida. Figures 1, 2 and 3 were drawn to the same scale; also figures 4 and 5.

INFECTIVITY TO CANARIES OF SPOROZOITES OF *PLASMODIUM RELICTUM* DEVELOPING IN VITRO*

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During the past several years, in a series of investigations carried out in this laboratory on the in vitro cultivation of the mosquito phase of *Plasmodium relictum*, it has been possible to carry the parasite in culture through a series of steps from the stage of gametocytes in the lumen of the stomach of the vector, *Culex tarsalis*, to the formation of mature oocysts containing viable sporozoites. The most recent of a series of reports of this work is that of Ball and Chao (1960). At the time that paper was published, it had not yet been possible to prove that the sporozoites which developed in vitro from cultured oocysts were infective, even though their viability was clearly demonstrable on the basis of motility.

After further trials, we have succeeded in producing normal infections in susceptible canaries by the injection of free sporozoites along with mature oocysts, both of which had developed in culture from oocysts that were in a somewhat advanced stage but still immature when they were removed from a mosquito.

MATERIALS AND METHODS

The general methods of culture have been described in previous papers (Ball, 1954; Ball and Chao, 1957; Ball and Chao, 1960). In the experiments described here, all of the cultures were carried in small Petri dishes 18 mm in diameter by 8 mm high (Chao and Moore, 1961). This type of container provided for ease of handling and also insured that the free sporozoites were concentrated in the relatively small volume of culture fluid which could be safely injected. If over 0.15 ml was given, the canary exhibited a state of shock, which was occasionally fatal.

The cultures in this series were maintained without change of medium. In some instances, they were aerated with a gas mixture of 5 percent CO_2 and 95 percent air. In one experiment, they were placed on a rocker. Some were maintained at room temperature; others at 22 C (table I). The composition of the medium was essentially that used by Ball and Chao (1960). The principal modification of this medium was the addition of a saline extract of whole pupae or of adults to some of the cultures. The concentration of mosquito extract in the final medium was approximately 0.2 percent.

Culex tarsalis were fed on canaries which showed gametocytes of *P. relictum* in their peripheral blood. When the oocysts resulting from this infection had reached a stage of considerable development but were still immature (stages 7a–8c as listed by Ball and Chao, 1960) (see also table I), the stomachs with attached oocysts were transferred to in vitro cultures as described above. From 24 to 96 hours later, some of the immature oocysts had become mature and contained sporozoites, or they had burst, releasing motile sporozoites. The sporozoites were picked up with a No. 23 hypodermic needle into a 1 cc tuberculin syringe, and injected intravenously through a 27-gauge needle into the leg of a clean canary. Immediately after this, mature oocysts plus a few free sporozoites remaining in the fluid were sucked up with a No. 23 needle and injected directly into the breast muscle of the same bird. Examinations for parasitemia were begun 5 to 10 days later and continued every other day until either gametocytes were present, or else a sufficient number of examinations indicated that no infection had occurred. In the latter case, birds were considered negative if no parasites had appeared in the blood 2.5 to 5 weeks after inoculation. In some instances, negative birds were tested further and found to be infectible following transfer of infective blood from positive canaries (table I).

Control infections produced by direct intravenous injection of sporozoites and by intramuscular inoculation of mature oocysts from infective mosquitoes showed parasitemia in 8 to 12 days (table I).

RESULTS

As shown in table I, the two positive infections (birds 1351 and 21) were produced from oocysts which had been cultured 48 or 96 hours, respectively; parasitemia was first noted on the 10th day with the 48-hour culture and on the 9th day with the 96-hour culture. This compares favorably with the length of time (8 to 12 days) for development of the blood phase in the controls. *C. tarsalis* fed on the birds infected with sporozoites which had developed in vitro had sporozoites in their salivary glands in the same

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TABLE I. *Results of injections of canaries with sporozoites and mature oocysts of* P. relictum *developed in vitro.*

Bird No.	Age, size & stage* of oocysts at beginning of culture	Days oocysts cultured	Conditions of culture	Infection in canary
63	13 days ; 65 microns, 8c, 10a	1	22 C	Negative 5–34 days Positive later from infected blood
28657	13 days ; 65 microns, 8c, 10a	2	Room temp.	Negative 10–25 days
27772	10 days ; 55 microns, 8b	2	22 C ; aeration with 5% CO_2-95% air	Negative 5–27 days
905	7 days ; 50 microns, 7b, 8a	3	3 stomachs at 22 C 1 stomach at room temp.	Negative 6–24 days Positive later from infected blood
268	7 days 8b	2	22 C ; aeration with 5% CO_2-95% air	Negative 9–23 days
1361	10 days ; 61 microns, 8b	1	Room temp. (26.5 C)	Negative 5–19 days Positive later from infected blood
1351	10 days ; 61 microns, 8b	2	Room temp. (26 C)	Positive 10th day Mosquito biting #1351 had sporozoites in salivary glands at 10 days
3032	5 days ; 44 microns, 7a	4	Room temp. aerated as above (1 culture) Rocked but not aerated (4 cultures)	Negative 5–25 days
559	9 days– 50 microns, 7a	3	22 C	Negative 6–25 days
9	9 days ; 50 microns, 7a	3	22 C	Negative 6–25 days
21	10 days ; 50 microns, 8a, 8b	4	22 C	Positive 9th day Mosquito biting #21 had sporozoites in salivary glands at 11 days
6426	10 days	—	—	Positive 12th day
7760	10 days	—	—	Positive 8th day
11938	10 days	—	—	Positive 8th day
1369	13 days room temp. 16 days at 4 C	—	—	Positive 8th day

* Designations 8a, 8b, etc. refer to photographs of stages so numbered in Ball and Chao, 1960. Stage 10a is that of a mature oocyst, but birds No. 63 and No. 28657, the only ones injected with this stage, were both negative.

incubation time as did mosquitoes fed on birds infected with parasites which had not been cultured.

Although the medium used was satisfactory for the development of sporozoites from immature oocysts, apparently it was not particularly suited for maintenance of free sporozoites since over half of them lost their motility and became deformed in 24 hours, and at 48 hours all activity was lost and practically none was normal in appearance. Whitman (1948) was able to maintain infectivity in sporozoites of *P. gallinaceum* taken from salivary glands and kept in extract of washed chicken erythrocytes for 72 hours, but not in sporozoites which had been in normal saline for 3 hours. Brackett and Hughes (1945) demonstrated that sporozoites of *P. gallinaceum* were injured after exposure to normal chicken serum in as short a time as 15 minutes at room temperature following removal from the salivary glands of the mosquito.

A further indication that our medium was favorable for developing oocysts was the fact that they continued to produce and discharge sporozoites for as long as 5 days after the mosquito gut had been transferred to culture.

The addition of mosquito extract made from whole pupae or adults crushed in normal saline, subsequently filtered, and added to the medium to produce a final concentration of approximately 0.2 percent apparently was of no value in extending the infectivity of the sporozoites. None of the cultures containing pupal extract was infective. Although one of the successful infections came from a culture containing extract of adult mosquitoes, the other culture had no mosquito extract of any kind added to it.

In general, aeration with a mixture of 5 percent CO_2 and 95 percent air was not favorable for the older stages of *P. relictum* from the mosquito. Aeration maintained the pH at 6.8 to 7.0, but in five cultures so treated, no sporo-

zoites were seen after 3 days, while 5 other cultures of the same material started at the same time but not aerated had many motile sporozoites. Since the medium did not maintain normal sporozoites for 48 hours, this difference was probably due to an inhibition in the aerated cultures either of the development or of the hatching of the oocysts. Possibly the alkaline pH of 7.9 in the non-aerated cultures was the factor responsible for the continued production of active sporozoites, but this is higher than the pH of normal hemocoele fluid of mosquitoes and of almost all other insects (Roeder, 1953; Hayes, 1953).

The older oocysts developed as well in culture at 26 C as at 22 C. This was also true for cultures which were rocked as compared with those that were not rocked.

DISCUSSION

The successful production in culture of infective sporozoites from immature oocysts completes an in vitro cycle of development of the mosquito phase of *P. relictum*, starting with the gametocytes in the stomach lumen of *C. tarsalis* (Ball and Chao, 1960). This cycle has been carried to completion, however, only by overlapping cultures started at different stages of development, individual cultures rarely continuing to show growth of the malarial parasite beyond 5 days. With all of the stages from the mosquito shown to be capable of culture, further advance will be in the direction of longer cultivation of individual preparations, leading it is hoped to development of a single culture from gametocyte to sporozoites.

The production of infection in only 2 of the 11 canaries injected is probably due in part to lowered viability of sporozoites in the culture medium as well as to the relatively few sporozoites injected. However, it should be noted that Majid and Sinton (1933) and Raffaele (1934) were unable to produce infection with *P. relictum* by intravenous injection into susceptible birds with sporozoites from 15 to 32 pairs of salivary glands. Wolfson (1936) obtained a good infection in only one out of three canaries injected intravenously with the sporozoites of salivary glands of two mosquitoes; one bird was negative, while another had very few infected erythrocytes. Warren and Coggeshall (1937), on the other hand, had success with *P. cathemerium* sporozoites injected intravenously. According to Rozeboom and Shaw (1934), at least 200 sporozoites are needed to produce infection in canaries with *P. cathemerium*.

The experiments reported here offer, it is hoped, final proof that sporozoites are infective when they are released from the oocyst and very probably also while still unreleased from the mature oocyst. Gambrell (1937), Jerace (1937), Sandichi (1937, 1938), and Brooke (1942) were unable to produce infection in birds by injection of mature oocysts or of sporozoites obtained directly from them. Possible reasons for these negative results are discussed in the preceding paragraph. On the other hand, Missiroli (1937), Neri (1937), Shute (1943) and Trembley, Greenberg and Coatney (1951) all obtained infections from injections of oocysts and/or sporozoites taken from the hemolymph or expressed from oocysts. Despite the convincing evidence from the work of the latter group of investigators, some objection has been raised that there was at least a possibility of contamination from sporozoites which had been released from the salivary glands either naturally or as a result of trauma due to dissection.

We believe that the experiments reported in this paper effectively dispose of this objection since the stomachs were dissected out of the mosquitoes at a time when the oocysts were still immature. Contact with the salivary glands was impossible since these had been discarded 24 to 96 hours before free sporozoites developed.

SUMMARY

Sporozoites of *P. relictum* which developed in culture from immature oocysts attached to stomachs of *C. tarsalis* were infective to susceptible canaries. In successful infections, the sporozoites injected were obtained from cultures begun with immature oocysts 48 to 96 hours previously. All stages of *P. relictum* in the mosquito from gametocyte to infective sporozoite have now been grown in vitro. These results offer additional evidence that sojourn in the salivary glands is not required for the sporozoites to become infective.

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INFECTIVITY AND PATHOGENICITY OF POLYXENIC AND MONOXENIC *ENTAMOEBA INVADENS* TO SNAKES KEPT AT NORMAL AND HIGH TEMPERATURES AND THE NATURAL HISTORY OF REPTILE AMOEBIASIS

E. MEEROVITCH*

In a preliminary note Meerovitch (1960) reported the inability of *Entamoeba invadens* from polyxenic cultures grown at room temperature to establish infections in snakes (*Thamnophis sirtalis*) kept at temperatures between 34 and 37 C. Barrow and Stockton (1960) reported on *E. invadens* infection of five species of snakes kept at 13 to 14, 15–28, 25, and 30 C. They found that in general no pathological changes were produced at 13 C, while at 25 C, *E. invadens* produced pathological changes of varying degrees of severity. Their snakes kept at 30 C (one of two *Natrix sipedon* and one *T. sirtalis* had severe lesions in the internal organs.

MATERIALS AND METHODS

Our preliminary experiments were repeated, using a larger number of *T. sirtalis* than in the earlier experiments. The same strain of *E. invadens* (I.P.2) was also used. The amoebae were obtained from stock cultures routinely incubated at 25 and 35 C in Balamuth's egg yolk infusion medium with an undetermined bacterial flora, and in modified Shaffer-Frye medium with a penicillin-inhibited *Bacteroides* sp. as the only bacterial associate.

The monoxenic cultures of *E. invadens* in the modified Shaffer-Frye medium were established from amoebae grown axenically in Stoll's liver broth-raw liver extract medium. The amoebae grow extremely well in the modified Shaffer-Frye medium but encystation in this medium is only sporadic.

By gradual serial transfer through 30 and 33 C, adaptation of amoebae to growth at 35 C was accomplished. Growth at 35 C in both media is characterized by a considerably lower population density than at 25 C, bigger organisms, complete lack of encystation, and a shorter life of the culture, making weekly transfers mandatory. Attempts to transfer the 35 C-adapted amoebae to 37 C were unsuccessful; it appears that 35 C is the highest temperature to which *E. invadens* may be adapted in vitro.

The snakes, *T. sirtalis*, used in these experiments were captured on Manitoulin Island in Lake Huron, Ont. Each snake was kept individually in a glass jar. Fecal examinations were made periodically to determine their freedom from parisitic amoebae. With the aid of a stomach tube the snakes were force-fed with a mixture of raw egg, milk, and cod liver oil. A stomach tube was also used for inoculating the snakes with amoebae. In experiment No. 1 each snake received approximately 200,000 amoebae and in experiment No. 2, approximately 500,000 amoebae. In the case of polyxenic amoebae incubated at 25 C the numbers inoculated included both trophozoites and cysts. After inoculation each snake was kept in an individual glass jar, some at room temperature (20 to 25 C), and some in a walk-in incubator at 35 C.

When snakes died or were killed the gross condition of their internal organs was noted. Portions of the liver and large intestine were placed in Balamuth's medium and adjacent portions of the same organs fixed for histological study.

RESULTS

Infection experiments Nos. 1 and 2 are summarized in tables I and II, respectively, in which all data pertaining to snakes kept at 35 C are grouped together, as are those pertaining to the snakes kept at room temperature. From these tables it will be noted that amoebae were not always found on histological examination of tissues from the snakes, although in some cases cultures from the same tissues eventually became positive for amoebae. This must have been due to the presence of amoebae in such small numbers that they were either absent or undetected in the sections examined.

The reasons why snakes B1 and B2 in experiment No. 2 remained uninfected and died within 2 days of inoculation are unknown; this might possibly be attributed to the numbers of organisms with which they were inoculated. The author's previous experience has been that all snakes infected with polyxenic *E. invadens* and kept at room temperature developed typical amoebiasis.

The establishment of a transient intestinal infection at a high temperature such as exhibited by snake A5 has been noted previously (Meerovitch, 1960).

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TABLE I. (*Experiment 1*) *Infection of* T. sirtalis *with* E. invadens *from polyxenic cultures grown at 25 and 35 C.*

Snake No.	Amoebae at degrees C	Snakes at degrees C	Dead or killed after (days)	Culture		Histological examination			
						Intestine		Liver	
				Intestine	Liver	Pathology	Amoebae	Pathology	Amoebae
A1	35	35	D 15	–	–	none	not seen	none	not seen
A2	35	35	D 5	–	–	none	not seen	none	not seen
A5	25	35	D 6	+	+	none	not seen	none	not seen
A6	25	35	D 20	–	+	none	not seen	none	not seen
A3	35	20–25	K 23	+	+	eosinophilia	in musoca	none	not seen
A4	35	20–25	K 23	+	+	typical pathology	in tissues	hemorrhagic	not seen
A7	25	20–25	K 23	+	+	typical pathology	in tissues	hemorrhagic	in blood vessels and parenchyma
A8	25	20–25	K 23	+	+	typical pathology	in tissues	hemorrhagic	in blood vessels and parenchyma

DISCUSSION

These experiments have confirmed our earlier finding that the pathogenicity of *E. invadens* in snakes kept at high temperatures is either eliminated or greatly reduced. None of the snakes in our preliminary experiments became infected at higher temperatures while some did in the present series of experiments; the reason is that the temperature of the hot room used in the preliminary experiments went as high as 37 C, while in the experiments described here it was kept steady at 35 C.

It can be concluded that although *E. invadens* may be infective to snakes kept at 35 C, such infections are never as pathogenic as at room temperatures. The death of snakes kept at 35 C was not due to amoebiasis but probably to excessive warmth and dehydration. Lueth (1941) found the lethal temperature for *Thamnophis radix* to be 41 C.

Monoxenic amoebae are infective and moderately pathogenic to snakes kept at room temperature but not infective at 35 C.

E. invadens, grown in either polyxenic or monoxenic cultures at 35 C, are infective and pathogenic to snakes kept at room temperature, but the monoxenic amoebae are less pathogenic than the polyxenic.

It was originally suggested (Meerovitch, 1960) that adaptation of *E. invadens* to in vitro growth at 35 C would be followed by its ability to infect snakes at this temperature

TABLE II. (*Experiment 2*) *Infection of* T. sirtalis *with* E. invadens *from polyxenic and monoxenic cultures grown at 25 and 35 C.*

Snake No.	Amoebae at degrees C	Kind of culture	Snakes at degrees C	Dead or killed after (days)	Culture		Histological examination			
							Intestine		Liver	
					Intestine	Liver	Pathology	Amoebae	Pathology	Amoebae
B3	25	poly.	35	K 32	+	+	none	not seen	none	not seen
B4	25	poly.	35	D 5	+	+	none	few in lumen	none	not seen
B7	35	poly.	35	D 21	–	–	none	not seen	none	not seen
B8	35	poly.	35	D 12	–	–	enteritis	not seen	none	not seen
B11	25	mono.	35	D 2	–	–	none	not seen	none	not seen
B12	25	mono.	35	D 9	–	–	none	not seen	none	not seen
B15	35	mono.	35	D 13	–	–	none	not seen	none	not seen
B16	35	mono.	35	D 21	–	–	none	not seen	none	not seen
B1	25	poly.	20–25	D 2	+	+	slight erosion of mucosa	in lumen, few in mucosa	none	not seen
B2	25	poly.	20–25	D 2	–	–	none	not seen	none	not seen
B5	35	poly.	20–25	K 32	+	+	eosinophilia, inflammation	in lumen	hemorrhagic	not seen
B6	35	poly.	20–25	D 23	+	+	typical pathology	in tissues	hemorrhagic	in vessels and parenchyma
B9	25	mono.	20–25	K 32	+	+	mucosa and submucosa eroded	in tissues	small abscesses	not seen
B10	25	mono.	20–25	K 32	+	+	mucosa eroded	in tissues	hemorrhagic	not seen
B13	35	mono.	20–25	D 4	+	+	mucosa eroded, hemorrhage	in lumen	none	not seen
B14	35	mono.	20–25	K 32	+	+	none	in lumen	none	not seen

with the typical severe results. This supposition was not confirmed. Our experiments have demonstrated that the degree of invasiveness and pathogenicity is determined by the temperature at which the hosts are kept.

The present study has shown that *E. invadens* is not obviously pathogenic at 35 C. The presence of amoebae (determined by culture) in the apparently normal livers of snakes kept at 35 C and infected with polyxenic amoebae grown at 25 C is highly significant and must be regarded as an indication of pathogenicity. This points to the extreme susceptibility of snakes to this amoebic infection, as opposed to the commensal relationship between *E. invadens* and herbivorous Chelonia (Meerovitch, 1957, 1958a, 1958b).

The effect of concomitant bacteria was demonstrated by comparing the results of infections with polyxenic and monoxenic amoebae grown at 35 C in snakes kept at room temperature. The results, as seen in table II, show clearly that monoxenic amoebae are less pathogenic than polyxenic amoebae. Although no control infections were made using the bacteria alone, examination of the results of infections in snakes kept at room temperature with polyxenic and monoxenic amoebae grown at 25 C shows that there was no significant difference in the pathology produced by the two kinds of amoebae.

Barrow and Stockton (1960) suggest that snakes which normally occupy subterranean chambers where the temperature may be in the range of the lower temperatures used in their studies (i.e., 13 to 14 C) may be infected with *E. invadens* without any pathogenic effect. Because they have not observed any cysts in their snakes kept at these low temperatures, they postulate that cyst production (which may even be seasonal), may be influenced by some still undetermined environmental factor which was not present under their experimental conditions. Their conclusion regarding lack of tissue invasion by the amoebae at low temperatures is based on gross and microscopical examination of the tissues. As already mentioned, some of our snakes which had latent infections also did not show any macro- or microscopical lesions, although their livers were invaded by amoebae.

Some information has been obtained in this laboratory on the behaviour of polyxenic *E. invadens* in vitro at 15, 12.5, 10, 7.5 and 5 C, (Lachance, unpublished data). Two strains tested survive, but do not multiply at temperatures below 12.5 C. Some encystation occurs at 15 C, but generally not at 12.5 C. At low temperatures the amoebae are large, sluggish and do not ingest starch.

The observations of Barrow and Stockton (1960) and our own indicate that *E. invadens* in snakes behaves in a similar manner at temperatures which are well below and above its optimum.

To postulate that snakes may be normally parasitized by *E. invadens* at low temperatures is to assume that this parasitism is limited only to the snakes' periods of inactivity. Temperatures of 13 C or thereabouts may be encountered only in the temperate regions. Snakes are essentially tropical and subtropical animals and relatively few species are found in the temperate fringes of their distribution. It is known that snakes in temperate regions during the summer feed voraciously in order to compensate for their period of hibernation. Thermoregulation in reptiles by means of surface absorption of solar radiation and by elimination of heat from the skin, as studied by Cowles (1958) and observed by Lueth (1941) and Carpenter (1952), helps to render snakes' body temperature more constant than that of the environment. This mechanism cannot maintain the body temperature at levels far above or below that of the environment. Consequently, in the temperate regions in the summer, the snakes' body temperature during periods of their maximum activity must be relatively high.

If it is assumed that *E. invadens* infection of snakes is limited to periods of hibernation, it would mean that the infection is acquired in hibernacula. This would require the ingestion of cyst-containing substrates. There would appear to be only three methods by which the snake endoparasites gain entry: (1) through the skin through arthropod vectors, (2) by direct penetration (for example, *Kalicephalus* (Schad, 1956)), or, (3) by ingestion of intermediate hosts. As far as is known, no snake endoparasites gain entry directly through the mouth. While *E. invadens* infection of snakes by means of transport hosts such as earthworms might be a possibility such a mode of infection would necessitate a copious seeding by the snakes of the substrate with amoebic cysts. Because there are no records of natural infections of snakes with *E. invadens*, the transport host hypothesis

appears untenable. We have examined many snakes captured in early spring soon after their emergence from hibernation but have never found *E. invadens* in any of them.

The fact that no parasitic amoebae are known to be natural parasites of carnivorous animals is significant. Herbivores, from reptiles to mammals, harbor commensally a number of species of parasitic amoebae. *E. histolytica* is most likely a natural parasite of Old World monkeys. Kittens have been used a great deal as experimental hosts of *E. histolytica* because of their high susceptibility to tissue invasion by that amoeba. In our opinion, the relationship of *E. histolytica* to kittens is very similar to that of *E. invadens* to snakes: neither of these animals is a natural host of the amoebae and consequently lack effective defence mechanisms against them.

Our previous findings together with experimental results reported here (particularly liver invasion in snakes at 35 C) make it conclusive, in our opinion, that natural infections of snakes with *E. invadens* do not occur. "Naturally infected" snakes become so through water and utensil contamination in captivity.

SUMMARY

1. *E. invadens* grown in polyxenic culture at 25 C are mildly infective to *Thamnophis sirtalis* kept at 35 C. Those grown in polyxenic culture at 35 C are just as pathogenic to snakes kept at room temperature, as are those grown at 25 C, but are not infective to snakes kept at 35 C.

2. *E. invadens* grown in monoxenic culture at 25 C are not infective to snakes kept at 35, but are infective and pathogenic at room temperature. Those grown in monoxenic culture at 35 C are not infective to snakes at 35 C but are moderately infective at room temperature.

3. Biological and ecological considerations regarding snakes and *E. invadens* are presented to show that *E. invadens* does not infect snakes in nature.

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KHAWIA IOWENSIS N. SP. (CESTODA: CARYOPHYLLAEIDAE) FROM *CYPRINUS CARPIO* L. IN IOWA

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Caryophyllaeid tapeworms of the subfamily Lytocestinae Hunter, 1927, have been reported from fresh-water fish in Africa, Asia, Europe, Australia, and North America. Only one species of this subfamily, namely *Atractolytocestus huronensis* Anthony, 1958, has been described from North America.

When Rehder (1959), during his study on carp (*Cyprinus carpio* L.) in the Des Moines River, Boone, Iowa, collected a caryophyllaeid tapeworm, he tentatively classified it as *Caryophyllaeus laticeps* Pall. This species was previously recorded from *Cyprinus carpio* and *Abramis brama* in Europe. Closer examination of Rehder's specimens and of additional worms collected from the same area, however, indicated that the specimens at hand could not be considered as belonging to the genus *Caryophyllaeus*. Examination of sections revealed that the preovarian vitellaria were located in the cortical parenchyma, a characteristic of the subfamily Lytocestinae. In the subfamily Caryophyllaeinae, to which *Caryophyllaeus* belongs, the vitellaria are located in the medullary parenchyma.

Careful comparison of wholemounts and sections indicated that the specimens exhibited characteristics typical of the genus *Khawia* Hsü, 1935. They could not, however, be ascribed to any known species of that genus and are consequently considered as constituting a new species, *Khawia iowensis*, to be described in detail below.

According to Yamaguti (1959) the order Caryophyllidea Ben. in Olsson, 1893, includes but a single family, Caryophyllaeidae Leuckart 1878, subdivided into three subfamilies (Caryophyllaeinae Nybelin, 1922, Lytocestinae Hunter, 1927, and Capingentinae Hunter, 1927). Members of the subfamily Lytocestinae are parasites of clariid, mormyrid, siluroid and cyprinid fishes. There are at present 12 genera in this subfamily including *Khawia*, erected by Hsü (1935) when he described *Khawia sinensis* from *Cyprinus carpio* in China. Five additional species are now known: *Khawia* (= *Caryophyllaeus*) *japonensis* Yamaguti, 1934, from *Cyprinus carpio* in Japan, *Khawia* (= *Bothrioscolex*) *prussicus* Szidat, 1937, from *Carassius carassius* in Germany, *Khawia* (= *Bothrioscolex*) *rossittensis* Szidat, 1937, from *Carassius carassius* in Germany, *Khawia* (= *Bothrioscolex*) *dubius* Szidat, 1937, from *Carassius carassius* in Germany and *Khawia baltica* Szidat, 1941, from *Tinca tinca* in Germany.

A translation of Hsü's original French description of the genus follows:

Khawia. Lytocestinae. Scolex frilled at anterior end. Sexual ducts enter into a common genital atrium. Parenchymal musculature in single layer. Ovary H-shaped. Uterine convolutions extend well to anterior end of ovary. Uterine glands present. Vitelline follicles separated into preovarian and postovarian, the former always found in cortical parenchyma and the latter in both cortical and medullary parenchyma.

Parasites of Cyprinidae.

METHODS AND MATERIALS

This study is based upon the examination of 338 carp collected between October 1959 and October 1960 from waters within and bordering the State of Iowa. Carp examined ranged in size from 1.5 to 22 inches in total length.

Fish were collected by use of seine, hoop net, electric shocker, and by angling. Additional specimens were obtained from collections made by the rough-fish crew of the Iowa State Conservation Commission. Details of all collections are given in table I.

Two main study areas were involved during the course of the investigation. The first study area consisted of a 6-mile region of the Iowa River between Iowa Falls and Alden, Iowa. Fish were collected here at several times between October 1959 and October 1960. Angling and use of a hoop net provided the best means of securing specimens. Details of the Iowa River collections are given in table II.

Studies during the summer of 1960 were conducted at the Iowa Lakeside Laboratory, Milford, Iowa. Here, the main study area was the Little

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Sioux River. A hoop net was set at various points within a 7-mile length of river. The net remained in the river almost continuously from the middle of June until the last of August. Fish were removed 2 or 3 times daily. Fish were also secured by angling, and smaller specimens were taken with a seine. Details of the Little Sioux River collections are also given in table II.

TABLE I. *Summary of fish examined and degree of infection.*

Collection area	No. fish examined	No. fish infected	Percent of fish infected
Des Moines River			
Boone Co., Iowa	17	8	47
Iowa River			
Hardin Co.,Iowa	83	43	52
Izaak Walton Lake			
Story Co., Iowa	6	0	0
Lake East Okoboji			
Dickinson Co., Iowa	12	7	58
Lake Minnewashta			
Dickinson Co., Iowa	5	3	60
Lake West Okoboji			
Dickinson Co., Iowa	25	14	56
Little Sioux River			
Dickinson Co., Iowa	134	72	54
Mississippi River			
Des Moines Co., Iowa	34	2	6
Skunk River			
Story Co., Iowa	15	1	7
Swan Lake			
Dickinson Co., Iowa	7	0	0
Totals	338	156	44

Fish from Lakes West Okoboji, East Okoboji, and Minnewashta were secured from the State Conservation Commission. Collections from these and the other areas listed in table I were made only sporadically and are not included in the data presented in table II.

In most cases, fish were taken to the laboratory and examined immediately. The gut of the fish was cut open along its entire length and larger worms were removed and placed in saline. The gut was then cut into transverse sections, placed in saline, and examined under low power of a dissecting microscope for recovery of additional worms. The gut and its contents were then allowed to stand for approximately 30 minutes and were again examined.

TABLE II. *Collection data from the Iowa and Little Sioux Rivers.*

Collection area	Date	No. carp examined	No. fish infected	Percent infected	Maximum no. worms in one fish
Iowa River	Oct. 1959	3	2	67	55
	April 1960	20	5	25	3
	May 1960	12	3	25	1
	June 1960	4	3	75	4
	July 1960	13	5	38	18
	Aug. 1960	23	18	78	624
	Sept. 1960	8	7	88	96
Little Sioux River	June 1960	10	5	50	7
	July 1960	62	26	42	60
	Aug. 1960	57	37	65	70

Gravid worms, when placed in distilled water for an hour prior to fixation in AFA (alcohol, formol, acetic), increased considerably in length. Stained specimens of worms treated in this way showed details of internal structure much more clearly than did those prepared from flattened or unflattened specimens, which had been fixed in AFA immediately upon removal from the host.

Specimens for wholemounts were stained in Mayer's paracarmine with fast green counterstain, dehydrated, cleared in terpineol or xylene and mounted in various resinous media. Cross, sagittal, and frontal sections were used in the study of internal structures. Worms for sectioning were embedded in paraffin and cut at 12 microns. Sections were stained with Harris' haematoxylin and triosin.

To facilitate shedding of eggs, gravid worms were placed in either distilled water, tap water, or lake water. Eggs were shed in all three media. Since more eggs were shed in distilled water, this medium was used exclusively in later studies. For developmental studies, eggs were maintained in small stender dishes containing river water which had been boiled and aerated. Cultures were kept at room temperature. Since Anthony (1958) reported that eggs of *Atractolytocestus huronensis* were continually destroyed by bacteria and mold when kept at room temperature, one or two drops of strong formalin were added to the first egg cultures. One control culture containing no formalin was also established. Development of oncospheres could be followed only in this control culture, although bacteria and mold were abundant. In subsequent cultures, no attempts were made to inhibit microorganisms but culture water was changed at one or two-day intervals.

All drawings were made with the aid of camera lucida or microprojector.

Adults

Khawia iowensis n. sp. With the characters of the genus. Gravid adults 17 mm (11 to 26) by 1.6 mm (1.0 to 2.1). Scolex frilled, somewhat wider than body. Neck indistinct. Body oval, flattened dorso-ventrally. Parenchymal musculature poorly developed.

Testes numerous, averaging 380 (328 to 490), extending from neck region to cirrus pouch. Testes spherical or elliptical, averaging 0.26 by 0.18 mm. Vas deferens convoluted anterior to cirrus pouch. Cirrus pouch large, measuring 0.66 by 0.61 mm. Seminal vesicle lacking. Common genital atrium present, located approximately 4.3 mm from posterior of worm.

Vitellaria averaging 0.19 by 0.13 mm, elliptical in shape, beginning anteriorly at about same level as testes. In region from neck to cirrus pouch, vitellaria cortical, encircling entire body. In region of uterine folds and ovarian wings, vitellaria cortical, but limited to lateral bands. Post-ovarian vitellaria

present, in both cortical and medullary parenchyma. Ovary H-shaped, approximately 2.0 mm in length, entirely medullary. Uterus not extending anterior to cirrus pouch. Uterine glands present. Uterus uniting with vagina to form utero-vaginal canal. Vagina expanding into large seminal receptacle in region of ovarian isthmus. Eggs measuring 54 by 42 microns, with spinose shell.

Parasitic in stomach and intestine of *Cyprinus carpio* and infrequently in intestine of *Ictiobus cyprinellus*, in Iowa.

Holotype: U.S.N.M.Helm. Coll., No. 39067, Beltsville, Md.

Paratypes: U.S.N.Helm. Coll., No. 39068, Beltsville, Md.

In several whole mounts of *K. iowensis* the typically frilled scolex was inverted, forming a pit. Szidat (1937) described such a pit as characteristic of the scolex of the genus *Bothrioscolex*. However, the scolex of *K. iowensis* exhibits its characteristic flattened appearance when attached to the host gut (fig. 18). This condition is quite different from that described by Szidat for *Bothrioscolex*.

The body of *K. iowensis* is slightly swollen in the region between the cirrus pouch and the posterior of the body. The terminal excretory bladder, in fixed specimens, is elongate, measuring 0.11 by 0.18 mm, and opens into an identation at the posterior of the body. Ten excretory ducts empty into the bladder.

The cuticle is approximately 7 microns thick. Longitudinal fibers are more prominent than circular fibers in the poorly developed parenchymal musculature. Dorso-ventral muscle fibers are present in the medullary region. Four longitudinal muscle strands are prominent in the neck region.

Testes, usually obscured by vitellaria and appearing in two longitudinal rows, begin near the anterior end. These rows are more distinct in young worms. Posteriorly, such rows become indistinct. In single cross sections, two to six testes usually appear in a single median row (fig. 3). Occasionally, additional testes are present, which in cross section lie dorsal or ventral to this median row. The convoluted vas deferens measures approximately 0.12 mm where it enters the muscular cirrus pouch. The male genital pore opens into a small, ventral, common genital atrium. The utero-vaginal aperture also opens into this atrium just posterior to the male opening. Neither an external nor internal seminal vesicle is present.

Vitellaria begin in the neck region, appearing as two lateral bands. The vitellaria soon become more extensive, forming a layer completely encircling the testes (fig. 3). This encircling layer continues to the level of the cirrus pouch. In the region of uterine folds, vitellaria appear as dense lateral bands of follicles. More posteriorly, a varying number of follicles is found lateral to each ovarian wing. In a single cross section, taken anterior to the cirrus pouch, approximately 17 follicles are present.

Preovarian yolk ducts appear as a pair of parallel ducts on each side of the body between the cirrus pouch and ovarian isthmus. The ducts are medial to the lateral band of vitellaria. Just anterior to the ovarian isthmus and medial to the ovarian wings, these paired ducts on each side of the body unite so as to form a single right preovarian and a single left preovarian duct. These single ducts now continue posteriad, dorsal to the ovarian isthmus. Just posterior to the ovarian isthmus, each preovarian duct unites with a postovarian duct, which is situated medial to the posterior ovarian wing. The right and left vitelline ducts thus formed meet in the midline and a common vitelline duct proceeds posteriorly a short distance before entering the ootype.

The isthmus of the H-shaped ovary lies ventrally in the medullary parenchyma. The oocapt lies medially on the posterior border of the ovarian isthmus. From this point, the oviduct extends caudally a short distance toward the right and is joined by a duct from the seminal receptacle. This fertilization duct then turns posteriorly to the ootype, which is surrounded by numerous cells of the Mehlis' gland. From the ootype, the uterine tract courses posteriorly to the anterior level of the postovarian vitellaria. Here it turns anteriorly and to the left, forming the uterus proper. The uterus describes several convolutions between postovarian vitellaria and ovarian isthmus before proceeding posteriad again to the posterior limit of the ovary. No uterine glands occur in this region of the uterus. The uterus now turns anteriad, passing to the right of the ootype and dorsal to the ovarian isthmus. The uterus describes regular folds between the ovarian isthmus and cirrus pouch. Well-developed uterine glands are associated with the uterus from the level of the postovarian vitellaria to the region of the cirrus pouch. In the immediate vicinity of the cirrus pouch, uterine glands are lacking. The uterus coils slightly in the region of the cirrus pouch and joins the

vagina to form the utero-vaginal canal approximately 0.56 mm posterior to the common genital atrium.

The vagina, situated ventral to the uterus, passes directly posteriad to the region of the ovarian isthmus. Here, it expands to form the large, elongate seminal receptacle, lying diagonally, dorsal to the ovarian isthmus. The seminal receptacle measures 0.27 by 0.69 mm.

Host-Parasite Relationships

In the present study, carp infected with *K. iowensis* varied in total length from 1.5 to 22 inches. The smallest worm recovered measured 0.64 mm in length (fig. 7). The greatest number of immature worms recovered from a single fish was 624. The greatest number of gravid worms taken from one host was 22.

K. iowensis is distributed throughout the entire length of the carp gut; however, immature worms are found most frequently in the stomach. Gravid adults usually occur in the first third of the intestine. The scolex of the worm is generally embedded in the mucosa of the gut (fig. 18), but in some cases both immature and gravid worms may be found completely detached. Immature worms attached to the stomach show great variation in the shape of the scolex (fig. 8). These variations are readily seen if the entire stomach is first fixed and the cestodes later removed.

The collection data tend to indicate that carp acquire their heaviest infections of *K. iowensis* in the late summer and early fall. Thus, in carp from the Iowa River, the incidence of infection and the maximum number of worms recovered from a single fish were greatest in August and September. Gravid worms were recovered only occasionally from April through August. Carp collected from Lakes West Okoboji, East Okoboji, and Minnewashta possessed heavier infections of immature worms as the summer progressed, but no collections later than August were made from these areas. Presumably, worms here too attain sexual maturity in late fall. This pattern of infection, however, was not so apparent in the Little Sioux River. Here, gravid worms were found regularly in carp during the months of June, July, and August 1960. No explanation is offered, on the basis of present knowledge, for this apparent discrepancy. Here too, however, the degree of infection and the number of worms per fish increased markedly as the summer progressed. It should be noted in table II that a very small number of fish were examined from both rivers during the month of June 1960. The percent of infection indicated from both areas at this time of year appears surprisingly high when compared with data for other months.

Anthony (1958) reported the same infection pattern for *Atractolytocestus huronensis* from carp in Michigan. Wunder (1939) found May to be the month of greatest infection of carp with *Caryophyllaeus laticeps* in Germany. His study was made on carp ponds where transfers of fish, lime treatment of ponds, and periodic drainage of the ponds probably affected the yearly infection cycle.

In the single collection of carp from the Mississippi River, a single immature tapeworm with a frilled scolex was found in each of two carp. No other caryophyllaeid with a frilled scolex has been described from the United States. The Iowa River, in which *K. iowensis* occurs, empties into the Mississippi River. Very probably, the worms from the Mississippi, too, are *K. iowensis*.

Further information on host-specificity of *K. iowensis* was obtained by examining fish whose feeding habits are similar to those of carp. Thus, only catostomid fish collected from the Iowa and Little Sioux Rivers were examined, since no other cyprinid fish of similar size and feeding habits to those of carp occur in these areas. Fifteen bigmouth buffalo (*Ictiobus cyprinellus* Le Seur) were examined, one of which harbored a single specimen of *K. iowensis*. Of the 48 redhorse examined, representing three species (*Moxostoma aureolum* Le Seur, *M. anisurum* Raf. and *M. erythrurum* Raf.) none harbored *K. iowensis*. Twelve white suckers (*Catostomus commersoni* Lacépède) and 24 carpsuckers representing three species (*Carpiodes cyprinus* Le Seur, *C. carpio* Raf. and *C. velifer* Raf.) were also examined; none harbored this parasite.

Oligochaetes, which are generally believed to be the intermediate host of caryophyllaeids, were consistently found in the gut contents of both carp and suckers from the Iowa and Little Sioux Rivers. These fish probably ingest a variety of larval caryophyllaeids. Host specificity of these worms must be explained on grounds other than selective feeding habits.

In September 1960, the Iowa State Conservation Commission poisoned the study area of the Iowa River as a rough fish control measure.

Further collections from this area were impossible. At the time of poisoning, over 100 carp were examined in the field in an attempt to secure eggs of *K. iowensis*. Specific counts of the number of worms per fish were not made, but the best estimate of the rate of infection was about 90 percent, with about 10 percent of the carp harboring one to five gravid worms. In several cases, hundreds of immature worms were attached to the stomach of a carp, a condition never prevailing in the spring and early summer.

Egg and Oncosphere

The first collection of eggs of *K. iowensis* was obtained from seven gravid worms taken from a single carp in the summer of 1960. Worms normally shed between 200 to 400 eggs after remaining in distilled water for 0.5 to 1 hour. Viable eggs are light brown in color, averaging 42 by 54 microns. The egg shell is covered with minute spines measuring approximately three microns in length. The eggs are operculate, but margins of the operculum are difficult to distinguish. However, on one occasion a fresh preparation of eggs was accidentally crushed, causing the opercula of two eggs to open (fig. 15). Normally, eggs when crushed do not open at the operculum, but split open at various points on the egg shell.

The eggs of *K. iowensis*, like those of most caryophyllaeids, are shed in an undeveloped condition. Eggs taken from the gut contents of carp are at the same level of development as those shed by worms in distilled water. Yolk cells, six to nine in number and measuring 6 to 8 microns in diameter, occupy most of the volume of the egg (fig. 11). Many small granules are scattered among the yolk cells. A zygote, measuring approximately 14 microns in diameter, is readily visible in sections of intrauterine eggs. There is no further development of the egg until it reaches water.

The embryo becomes clearly visible after the egg has been in culture 5 days (fig. 12). Its shape is very irregular and variable; no internal differentiation is evident in fresh preparations. At this stage, the embryo measures 21 microns in length. Between the 5th and 13th day, at which time the embryonic hooks appear, the oncosphere increases in size and assumes a definitely oval shape (fig. 13). The inner pair of hooks is slightly longer (12 microns) than either of the outer two pairs (9 microns). Two distinct, granular cells are found in the immediate vicinity of the inner pair of hooks. The only other differentiation visible in fresh preparations of the oncospheres are several globules, which appear to be oil droplets. No cilia were observed on the oncosphere at any time. The average time of oncosphere development is 15 days. The oval oncosphere usually lies along the long axis of the egg and measures 42 by 24 microns (fig. 14).

Movement of oncospheres is very limited, and is generally slow. On one occasion, however, oncospheres were observed to undergo rapid elongation and contraction, with some lateral movement. The hooks are also capable of movement. Oncospheres were never observed to emerge from the egg shell, nor were eggs with opened opercula found in cultures.

According to Sekutowicz (1934), the egg of *Caryophyllaeus laticeps* contains an oocyte and five to six yolk cells. Fertilization and cleavage do not occur until the egg reaches water. After 5 days in water, the embryo of *C. laticeps* measures 25 microns. Embryonic hooks appear between the 7th and 10th day. The oncosphere is fully developed after 14 days, and measures 40 to 65 microns in length. Two granular cells are shown, situated near the middle pair of hooks. In *K. iowensis*, the structure of the oncosphere and the course of its development appear to be quite similar to that of *C. laticeps*.

Sekutowicz kept embryonated eggs of *C. laticeps* in culture for more than 3 months but failed to observe emergence. In an attempt to observe emergence of oncospheres of *K. iowensis*, several oligochaetes from the streams under study were macerated and the water extract of these annelids was added to a preparation of embryonated eggs. Results were negative. Further studies on the life cycle are in progress.

DISCUSSION

Of the 12 genera comprising the subfamily Lytocestinae, only three possess postovarian vitellaria: *Atractolytocestus* Anthony, 1958, *Lytocestoides* Baylis, 1928, and *Khawia* Hsü, 1935. *Khawia* is differentiated from these two genera by (1) the nature of its scolex, (2) the relative number of testes, and (3) the position of the postovarian vitellaria. In *Lytocestoides*, the scolex is described by Baylis (1928) as ". . . short, conical, with longitudinal grooves, but devoid of locular depressions." The scolex of

Atractolytocestus is described by Anthony (1958) as ". . . unspecialized, may possess a terminal introvert." The scolex of *Khawia*, on the other hand, is frilled or pitted at the anterior end, but never possesses a terminal introvert. In *Khawia* and *Lytocestoides* the testes are described as numerous, while in *Atractolytocestus* they number from 6 to 18. In *Khawia* and *Atractolytocestus* the postovarian vitellaria are located either medullary or both medullary and cortical, while in *Lytocestoides* the postovarian vitellaria are limited to the cortical parenchyma. The tapeworm described in this paper clearly belongs to the genus *Khawia* on the basis of its postovarian vitellaria situated in the cortical and medullary parenchyma, as well as by its characteristically frilled scolex and the possession of numerous testes.

K. iowensis may be differentiated from other species of the genus (including *Bothrioscolex*) by the following key:

1a Posterior wings of ovary obscured by vitellaria 2
2a Vitellaria in uterine region in form of a weak lateral band, vitellaria larger than testes ..... *K. sinensis* Hsü, 1935
2b Vitellaria in uterine region in form of a conspicuous lateral band, vitellaria not larger than testes 3
3a Scolex flattened dorso-ventrally when relaxed, no vitellaria lateral to ovarian wings ..... *K. baltica* Szidat, 1941
3b Scolex conical when relaxed, vitellaria extending lateral to ovarian wings *K. rossittensis* Szidat, 1937
1b Posterior wings of ovary not obscured by vitellaria 4
4a Length of adult 5 to 6 mm *K. dubius* Szidat, 1937
4b Length of adult 10 mm or greater 5
5a Anterior wings of ovary more than twice the length of posterior wings *K. prussicus* Szidat, 1937
5b Anterior wings of ovary approximately same length as posterior wings 6
6a Testes, in cross section, in distinct dorsal and ventral layers, about 7 testes per layer *K. japonensis* Yamaguti, 1934
6b Testes, in cross section, primarily in single layer of 2 to 6 testes *K. iowensis* n. sp.

K. iowensis fits Hsü's (1935) generic diagnosis in all respects; however, one additional characteristic (not included in the generic diagnosis) is worthy of mention. The eggs of *K. iowensis* possess a spinose shell. No mention of spines has been made for the eggs of any of the other described species of the genus. Szidat (1937) described eggs of *K. rossittensis* from fresh preparations, but no spines were indicated. Szidat (1937) also described the eggs of *K. prussicus* from balsam preparations, but made no mention of spines. Yamaguti (1934) described eggs of *K. japonensis* from balsam preparations without indicating the presence or absence of spines. In Hsü's (1935) description of eggs of *K. sinensis*, no indication is given of the type of preparation used, nor are spines mentioned. In *K. iowensis*, on the other hand, spines are readily visible on the surface of viable eggs normally shed by worms placed in distilled water. Under low power of a compound microscope, individual spines are not visible, but their presence is indicated by a definite rough, pitted appearance of the egg shell. Under oil immersion, individual spines may be clearly seen (fig. 16). Examination of egg shells from which the contents have been removed by pressure, indicates that the spines occur in a seemingly regular pattern of distinct longitudinal rows (fig. 15). Examination of sections showing intrauterine eggs indicates the presence of spines in some cases, while in others, only the sculptured effect is visible. Spinose egg shells have been described in only one other genus of the subfamily Lytocestinae, namely *Djombangia* Bovien, 1926.

Although it appears that a spinose shell is distinctive of *K. iowensis*, this characteristic cannot be used in differentiating this species from others of the genus until more careful observations have been made on the latter.

The location of postovarian vitellaria, according to several workers, is said to vary within the genus *Khawia*. Thus, in *K. sinensis* according to Hsü (1935), the postovarian vitellaria are situated in both the cortical and medullary parenchyma. This is true also of *K. iowensis*. Yamaguti (1934), however, described postovarian vitellaria of *K. japonensis* as being entirely medullary. Examination of his illustrations of this region would seem to indicate, however, that at least some follicles occur in the cortical parenchyma. The position of postovarian vitellaria in *K. prussicus*, *K. rossittensis*, *K. dubius*, and *K. baltica* was not indicated by Szidat (1937 and 1941). Personal correspondence with Szidat indicates that the type specimens for these species, unfortunately, are no longer extant.

Because of the apparent differences among

genera and species of the subfamily Lytocestinae with regard to location of postovarian vitellaria, Johri (1959) erected a new family, Lallidae. This family included *Khawia* Hsü, 1935, and *Stocksia* Woodland, 1937, both of which possess vitellaria found in the cortical and medullary parenchyma. Johri retained the genus *Bothrioscolex* Szidat, 1937, in the family Lytocestidae (= Lytocestinae) partially on the basis of differences of the scoleces (frilled in *Khawia* and possessing a pit in *Bothrioscolex*). Fotedar (1958) and Yamaguti (1959), on the other hand, placed *Bothrioscolex* in synonomy with *Khawia*. Fotedar also stated that *K. prussicus* is probably synonymous with *K. japonensis*. In our opinion, however, the differences in the shape of the ovaries, together with other differences noted by Szidat (1937), would seem to indicate that *K. prussicus* and *K. japonensis* should be retained as separate and distinct species.

SUMMARY

A caryophyllaeid tapeworm, *Khawia iowensis* n. sp., is described from *Cyprinus carpio*, in Iowa. One specimen of this parasite was also recovered from *Ictiobus cyprinellus*. Infected carp varied in size from 1.5 to 22 inches. In a single carp, the maximum number of immature worms recovered was 624; the maximum number of gravid worms recovered from a single carp was 22. Heaviest infections occurred in late summer and early fall. Forty-four percent of all carp examined harbored the parasite. The spinose eggs are undeveloped when shed. Unciliated oncospheres develop after 15 days in culture, but do not emerge from the egg shell.

ACKNOWLEDGMENTS

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Explanation of Plates

Abbreviations: CP, cirrus pouch; CVD, common vitelline duct; E, egg; ED, excretory duct; GA, genital atrium; MG, Mehlis' gland; OD, oviduct; OT, ootype; OV, ovary; PM, parenchymal musculature; SR, seminal receptacle; T, testes; U, uterus; UG, uterine glands; UT, uterine tract; UVC, utero-vaginal canal; V, vagina; VD, vas deferens; VG, vitelline gland; VTD, vitelline duct.

Plate I

Figure 1. *Khawia iowensis*, gravid adult.
Figure 2. Gravid adult, reproductive complex.

Plate II

Figures 3 to 6. Cross sections through gravid adult.
All figures drawn to scale in figure 3.
Figure 3. Cross section at level anterior to cirrus pouch.
Figure 4. Cross section between cirrus pouch and ovary.
Figure 5. Cross section at level of ovarian isthmus.
Figure 6. Cross section at level of postovarian vitellaria.
Figures 7 to 10. Immature adults. All figures drawn to scale in figure 10.
Figure 7. Smallest worm recovered from carp.
Figure 8. Two worms removed from carp stomach after fixation. Note scolex variations.
Figures 9 and 10. Adults at later stages of development, but not yet mature.
Figures 11 to 15. Eggs of *K. iowensis* in developmental stages. All figures drawn scale in figure 11.
Figure 11. Freshly shed egg.
Figure 12. Egg after 5 days in culture.
Figure 13. Egg after 10 days in culture.
Figure 14. Egg after 15 days in culture; oncosphere completely developed.
Figure 15. Egg showing opened operculum and arrangement of spines.

Plate III

Figure 16. *Khawia iowensis*, photograph of egg. Note conspicuous spines.
Figure 17. Photograph of egg containing developed oncosphere.
Figure 18. Photograph of adult worm embedded in intestine of *Cyprinus carpio*. Sagittal section.

PLATE I

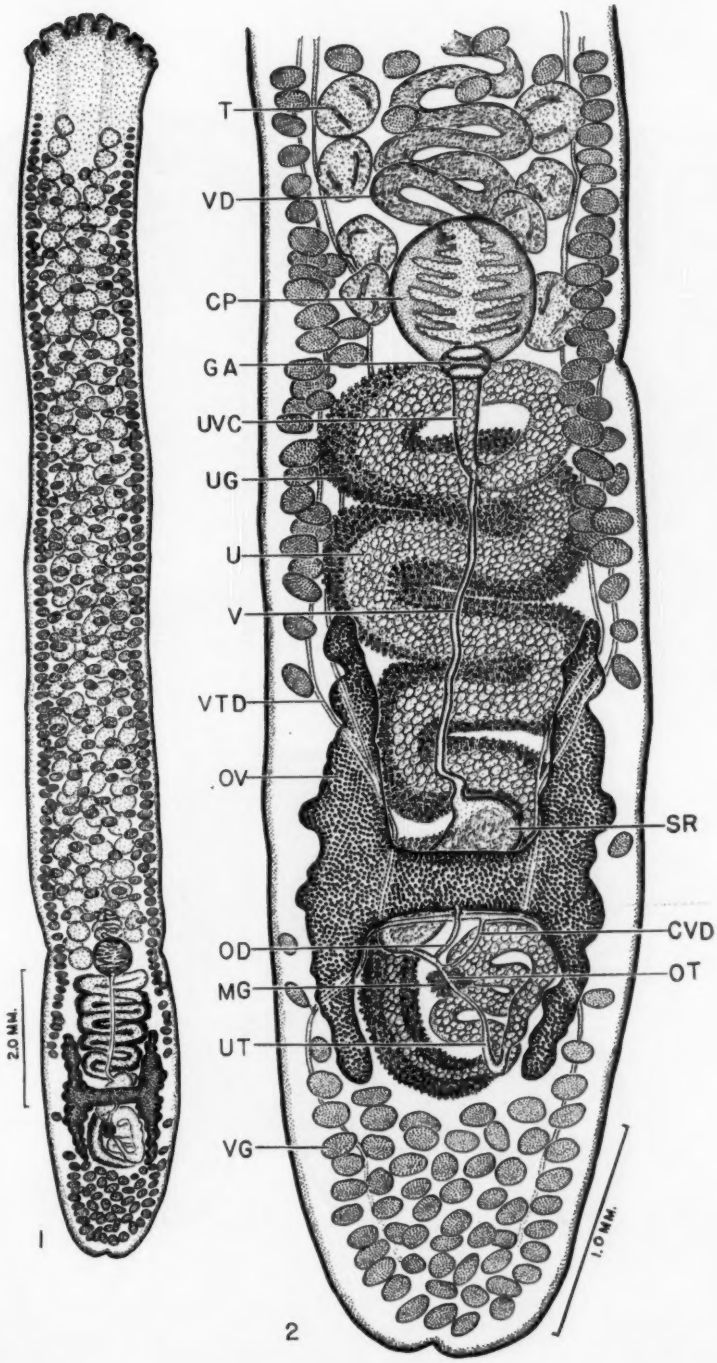


PLATE II

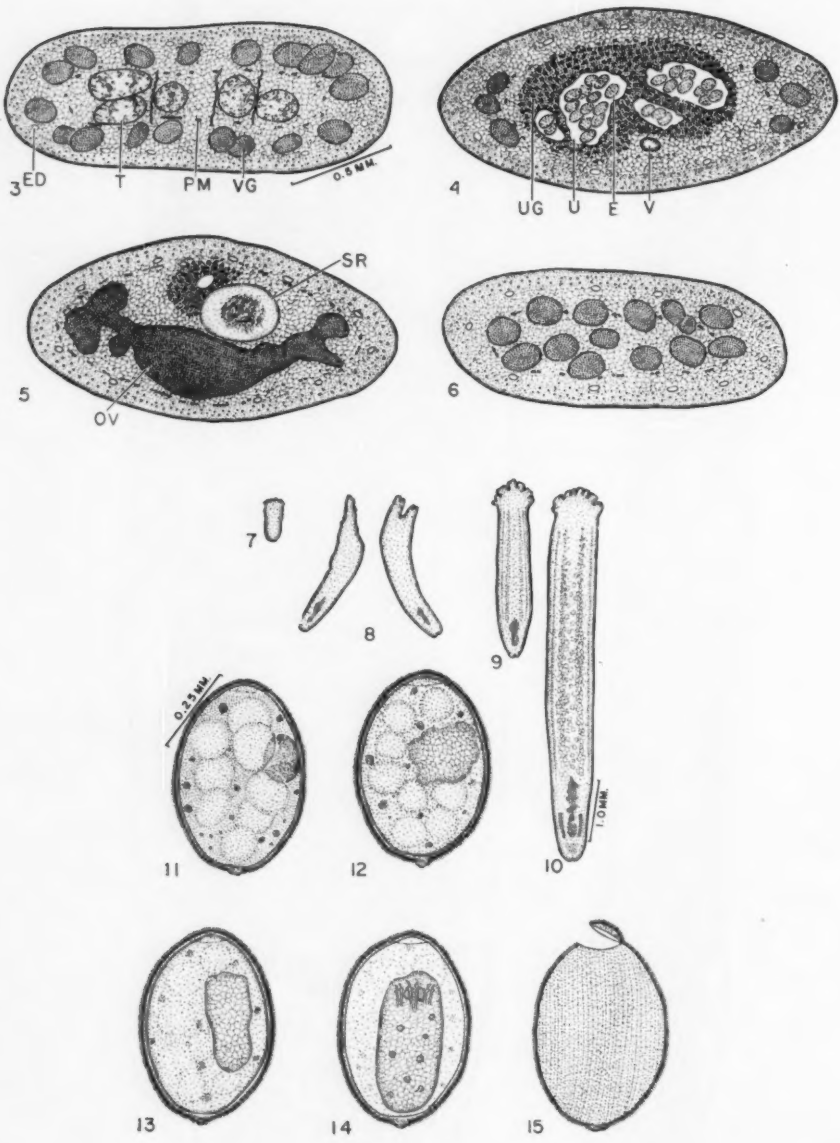
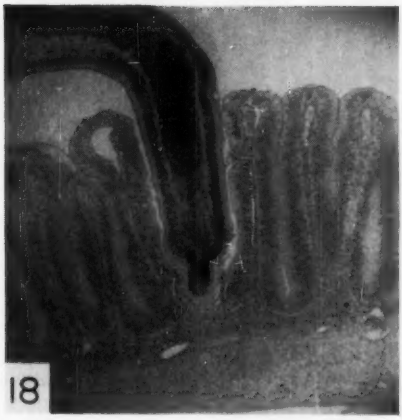


PLATE III



BAERIETTA IDAHOENSIS N. SP. A NEMATOTAENIID CESTODE FROM THE INTESTINE OF *PLETHODON VANDYKEI IDAHOENSIS* FROM NORTHERN IDAHO*

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During a survey of helminth parasites of amphibians and reptiles in Idaho in 1959, ten of 34 adult Couer d'Alene salamanders, *Plethodon vandykei idahoensis*, were found to be infected with a total of 22 Nematotaeniid cestodes. Most of the infected hosts harbored one or two worms, but one contained eight. The worms were identified as belonging to the genus *Baerietta* Hsü, 1935. Because they differ from the other described species of the genus, a new species, *Baerietta idahoensis* n. sp., is proposed.

MATERIALS AND METHODS

Hosts were examined as soon after collection as possible and immediately after sacrifice. Their intestinal tracts were opened in 0.7 percent physiological saline. The worms were removed, rinsed in saline solution, and fixed in A.F.A. under cover slip pressure. Whole mounts were stained in Harris' and Delafield's haematoxylin, and Semichon's acetic carmine. Serial sections were made from paraffin imbedded worms and stained with Harris' and Delafield's haematoxylin.

DISCUSSION

The following diagnosis is based on the study of 12 whole mounts, 5 sets of serial sections, and several pieces containing 3 to 5 gravid proglottids. All measurements are given in microns unless otherwise designated.

Baerietta idahoensis n. sp.

Description: Maximum length 24 mm, average 15.25. Scolex without hooks or rostellum, well defined, width 325 (302 to 336), length 221 (197 to 255); suckers 147 (132 to 164) in diameter. Diameter of neck 198 (174 to 244). Cuticula 5 microns thick, composed of 2 layers, having cuticular ridges with appearance of spines in whole mounts while in sections, outer portion appearing hairy. Genital anlagen first appear about 1.2 to 2.0 mm posterior to scolex; segmentation first evident in posterior half of worm where the uterus is well developed and most of the other organs have disappeared. A typical mature proglottid measures 139 in width by 37 in length; proglottids containing uterus with eggs measure 104 to 174 in width and 190 to 325 in length; terminal proglottids measure 418–441 in length and 158 to 166 in width; the maximum diameter of worms 239 (220 to 264). Testes, two, almost round in cross section 48 to 53 in diameter, cirrus pouch club-shaped, 51 to 58 in length; genital pores alternate irregularly. Ovary composed of large cells, round, 32 to 43 in diameter; vitelline gland spherical 26 to 30 in diameter. Uterus begins as small tube, becomes horseshoe shaped, and finally, when filled with eggs, assumes spherical shape, 69 to 87 in diameter, containing uterine capsules, with total of 12 to 20 eggs per uterus. Paruterine organs two, united basally, each containing 6 to 10 eggs. Eggs, including embryo shell measure 23.4 to 27.3 in length by 18.2 to 21.2 in width; embryo shell 1.3 to 1.5 thick; embryonal hooks well developed, central pair 12.0 to 13.0 long, lateral pairs 8 to 9 long.

Host: *Plethodon vandykei idahoensis* Slater and Slip, 1940.

Locality: Wolf Lodge Bay, Lake Couer d'Alene, Kootenai Co., Idaho.

Habitat: Small intestine.

Type: U. S. Natl. Mus. Helm. Col. No. 39084.

Discussion: The most prominent features that differentiate *Baerietta idahoensis* from all other species in the genus are the well defined scolex and slender strobila. It is slenderer than *B. baeri* Hsü, 1935, and has larger suckers, smaller testes, shorter cirrus sac. It is longer than *B. jägerskiöldi* (Janicki, 1928) and also has a smaller scolex, larger vitelline gland and uterus, less eggs per gravid segment, longer cirrus sac, and lesser diameter. *B. japonica* Yamaguti, 1938, has smaller suckers, thinner cuticle, a greater diameter and a shorter cirrus sac. *B. idahoensis* has a greater neck diameter than *B. janicki* (Hilmy, 1936) and also has a greater number of eggs, longer cirrus sac and is slenderer. It is longer than *B. diana* (Helfer, 1948) and also has larger suckers, smaller mature proglottid, ovary, and vitelline gland, greater number of eggs, and a thicker cuticle. It differs from *B. claviformis* Yamaguti,

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1954, in having a shorter length, smaller number of eggs, shorter embryonal hooks, and a smaller diameter. *B. montana* Yamaguti, 1954, is longer, has a larger scolex, suckers, greater neck diameter, larger testes, ovary, vitelline gland, and uterus, and is thicker. *B. enteraneidis* (Helfer, 1948) has a larger scolex, neck, a larger number of eggs and a greater diameter.

Douglas (1958) reviewed the Nematotaeniid cestodes and placed five species in the genus *Baerietta*. One of these species, *B. diana* (= *Proteocephalus diana*, Helfer, 1948), has been placed by Lehmann (1960) in *Cylindrotaenia*. Douglas' redescription of this worm (1958) describes two testes. The presence of two testes and the arrangement of the two paruterine organs shows that it belongs, as correctly placed by Douglas, in *Baerietta*. Douglas does not mention *B. claviformis* and *B. montana* (Yamaguti, 1954). He also omits *B. desmognathi* which was mentioned as a new species in an earlier abstract (Douglas, 1957). As he describes nothing but the early cleavage stages of the worm, the latter cannot be differentiated from other species in the genus and must be considered a *nomen nudum*. Yamaguti (1960) nevertheless lists this worm as belonging to *Baerietta* and also includes *B. enteraneidis* (= *Proteocephalus enteraneidis*, Helfer, 1948) as belonging to the genus *Baerietta*. The drawing by Helfer (1948: p. 361, fig. 6) shows six capsules in the gravid proglottid. This is not typical of *Baerietta*. He does not describe the testes of this worm, so it is impossible to place it in the correct genus.

Soler (1954) has placed *B. jägerskiöldi* (Janicki, 1928) in the genus *Nematotaenia* despite its having the typical *Baerietta* pattern of two testes and two paruterine organs. Soler presents a table giving measurements for this as well as several other species and cites Janicki as the observer. For *B. jägerskiöldi* she gives: length 15 mm, scolex diameter 550 microns, sucker diameter 183 microns. No bibliography is included in this paper, so it must be assumed that she is referring to Janicki's original description of the species (1928). In this, Janicki states ". . . von denen Keines die Länge von 10 mm. überstieg . . . Der Scolex ist sehr klein, er misst 0.149 mm. in Durchmesser; die 4 Saugnäpfe haben einen Durchmesser von je 0.062 mm." As Janicki's original description is in a source which is difficult to obtain, it is considered important to repeat it here.

SUMMARY

Baerietta idahoensis n. sp. from the small intestine of the salamander *Plethodon vandykei idahoensis* is described and is differentiated from the other known species of the genus. Discrepancies in the literature concerning the genus *Baerietta* are discussed.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the help of Miss Alice Boatright, Staff Artist, Department of Zoology, University of Illinois, who assisted in the preparation of the illustrations.

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Explanation of Plate I

All drawings made with the aid of a camera lucida.

Figure 1. *Baerietta idahoensis* n. sp., anterior end.

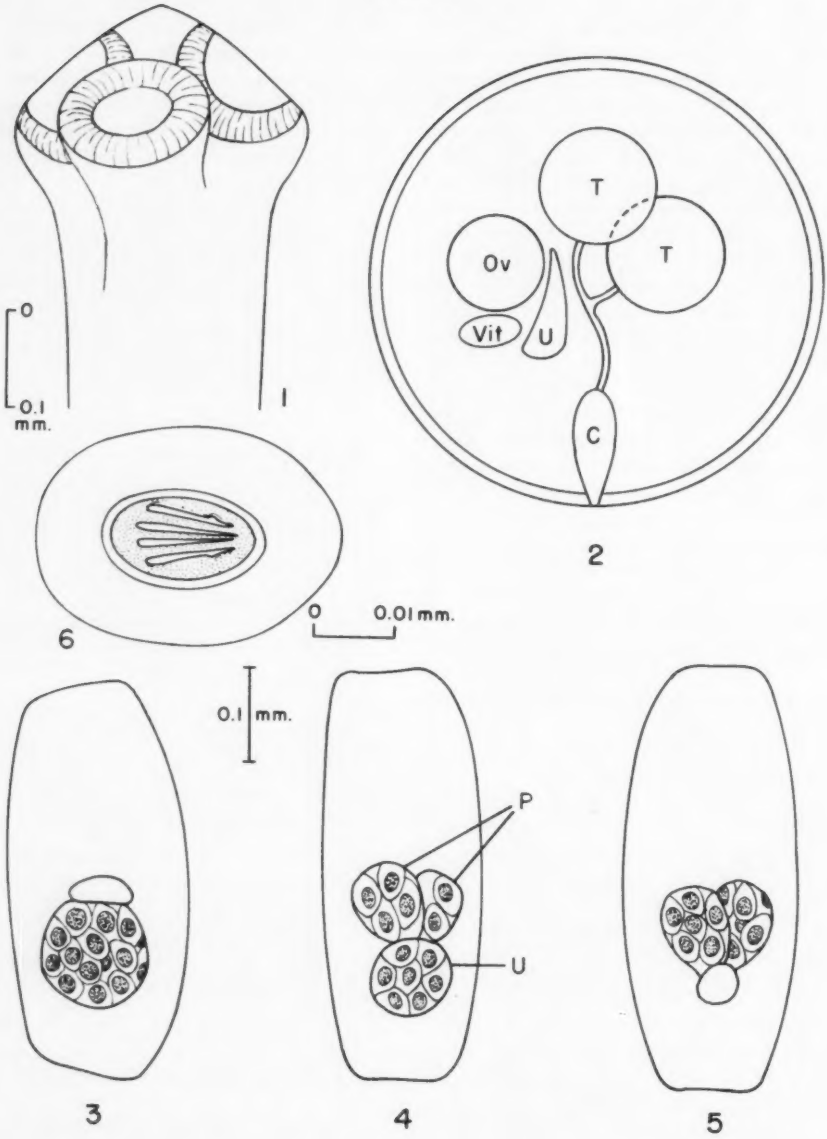
Figure 2. *Baerietta idahoensis* n. sp., diagram of cross section through mature proglottid.

Figures 3–5. *Baerietta idahoensis* n. sp., showing movement of eggs from uterus into the paruterine organ as one proceeds posteriorly along the worm.

Figure 6. *Baerietta idahoensis* n. sp., drawing of embryo showing hooks.

Abbreviations: C, cirrus sac; Ov, ovary; P, paruterine organ; T, testes; U, uterus; V, vitelline gland.

PLATE I



A DESCRIPTION OF ANOMALOUS AND ATYPICALLY DEVELOPED TAPEWORMS (PROTEOCEPHALIDAE: *CORALLOBOTHRIUM*) FROM CATFISHES (*ICTALURUS*)

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During a study of tapeworms of the genus *Corallobothrium*, two worms were found in Lake Texoma catfishes (*Ictalurus*) that were described as new species (Sneed, 1950). Jones, Kerley, and Sneed (1954, 1956) established a new subgenus Megathylacoides to accommodate these worms: *C.* (*M.*) *procerum; C.* (*M.*) *thompsoni;* and another species *C.* (*M.*) *tva* found by Jones and Kerley. Since some corallobothrid tapeworms of Lake Texoma catfishes exhibit atypical development and abnormal anatomy, only mature, normal individuals were used in the diagnosis of these species. However, it appears desirable to present a discussion of the development and seasonal occurrence of these worms, together with a brief description of the anomalous conditions in anatomy.

OBSERVATIONS

Seasonal Occurrence

During the winter months (October to April), Essex (1927) found only the plerocercoids of *Corallobothrium* in the catfishes of Illinois; these were small being 0.5 to 0.8 millimeter long. He stated that more advanced worms were not found in the catfishes of Illinois during these months.

Van Cleave and Mueller (1934) reported *C.* (*c.*) *fimbriatum* from catfish hosts, indicating that the size and degree of development depends somewhat upon the size and species of the host. These workers report that the worms from *Ictalurus punctatus* (channel catfish) were small and unsegmented, suggesting that this host was unsuitable for the worm. Most of their collections were made during the summer months, and little information was available on the cyclic occurrence of the worm.

Linton (1941) reported *C.* (*C.*) *fimbriatum* from *Ameiurus nebulosus* (*I. nebulosus*). His collections were made from May to September 1913 to 1918, inclusive. Some of his worms were as small as 3 millimeters and some as long as 50 mm. Their state of maturity or degree of cyclic occurrence was not discussed.

Corallobothria are very common in the catfishes of Lake Texoma, Oklahoma, during the winter. Extensive collections were made on February 27, 1948, April 2, 1948, October 31, 1948, February 28, 1949, and April 1, 1949. Additional collections were made in 1956, 1957, and 1958.

Three species were found: one was *C.* (*C.*) *fimbriatum* Essex, 1927; one, *C.* (*M.*) *thompsoni* Sneed, 1950; and the other, *C.* (*M.*) *procerum* Sneed, 1950. Although some plerocercoids of *C.* (*C.*) *fimbriatum* were recovered, most of the worms of this species were developed far beyond the plerocercoid stage. In fact, two worms collected on October 31, 1948, possess ripe proglottids. Many worms of this species collected on other dates had diverticula already formed in the uterus, but had no ripe proglottids. It is evident that this species grows to maturity during the winter in Lake Texoma. It appears, however, that maturity may be delayed when infection occurs during mid-winter or early spring, since worms collected in February and April are less mature than those collected in October. Also, it is possible that old worms are either eliminated from the host or the old strobilae are shed (and the scolices retained) some time in the late fall or winter. This statement appears plausible in view of Essex's investigations and my own findings.

The difference in the degree of maturity between *C.* (*C.*) *fimbriatum* in Oklahoma channel catfish and those of Illinois may possibly be related to the higher water temperatures in Oklahoma and to the different feeding habits of the host.

Atypical Development

C. (*M.*) *thompsoni* and *C.* (*M.*) *procerum* exhibit a very unusual type of developmental cycle. Many of the worms reach a length of 25 or 30 centimeters without developing mature

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proglottids. The ovary remains lattice-like in form, the uterus does not develop a cavity (occasionally a slight cavity appears), and no eggs are produced (fig. 1, B; for simplification, testes and certain accessory organs have been omitted from the drawings B). However, the proglottids become longer than broad. Leigh (1941) found cestodes of the genus *Raillietina* which exhibit the same atypical pattern of development.

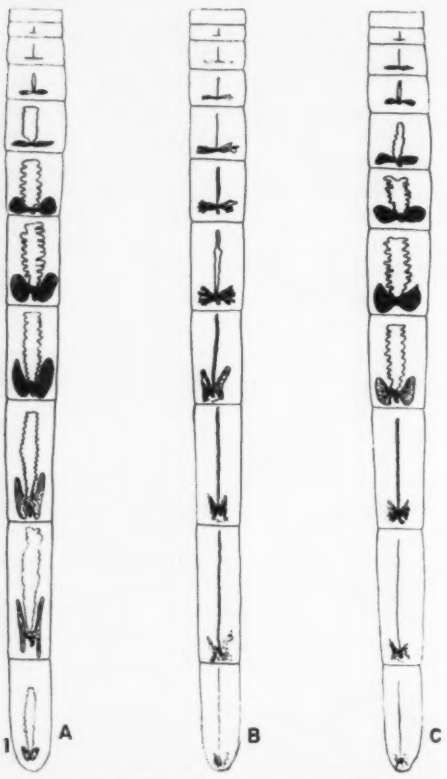

FIGURE 1. A typically developed strobilae of C. (*M.*) *thompsoni:* A, normal development; B, worm with no mature or ripe proglottid; C, worm with normally developing proglottids anterior and atypically developed or depleted proglottids posterior. (Testes and other accessory organs omitted.)

The series of worms in my collection from the channel catfish suggests that these atypically developed individuals are young, and that as the worms grow older the atypical segments are displaced posteriorly by normally developed proglottids. The irregular segments then wither away. Worms, which are of the same species, often taken from the same host at the same season of the year, exhibit three stages of development. One is atypical as described above (fig. 1, B). Another possesses mature and ripe proglottids which are more anterior in position than proglottids in which the uterus, ovary, etc., are still immature, or possibly physiologically incapable of ever producing sex products (fig. 1, C). Other worms possess the normal arrangement of immature and mature proglottids, i.e., the immature anterior, the mature posterior, followed by ripe segments (fig. 1, A).

Since all three types of worms were often collected at the same time of the year, it may be that this unusual development is related to the age of the worm rather than with the season. Extensive collections during the summer months would probably throw additional light on this point. Of course, it is equally possible that these worms are physiologically abnormal and will never produce normal proglottids.

These atypically developed worms, as well as many mature specimens, exhibit a wide variety of anomalous conditions, such as reversal of the sequence of mature and immature proglottids (as discussed above), a reduction or duplication of sex organs, bifurcated strobilae, twinning and split strobilae with various degrees of regeneration.

The normal, maturing proglottid of *C.* (*M.*) *thompsoni* is shown in figure 2; a normal, almost mature segment of *C.* (*M*) *procerum*, in figure 3.

Figure 4 illustrates the duplication of sex organs, a condition observed many times. In this proglottid, testes, vitellaria, and genitalia are duplicated, but the anterior ovary and uterus are incomplete. Several variations of duplicated organs were seen, some of which are illustrated in figures 5 and 6.

The anterior proglottid of figure 4 contains an extra cirrus organ on the left, which does not reach the exterior; there is no duplicated vagina on this side. On the right side there are both vagina and cirrus organs, but it appears that the vas deferens is rudimentary. The posterior segment of figure 5 also contains two sets of genitalia. The anterior cirrus organs is complete with vas deferens, but the vas deferens is incomplete in the posterior genitalia. Also, the vagina appears normal in the posterior set, but incomplete in the anterior set. Perhaps the presence of one exerts a retarding effect on the

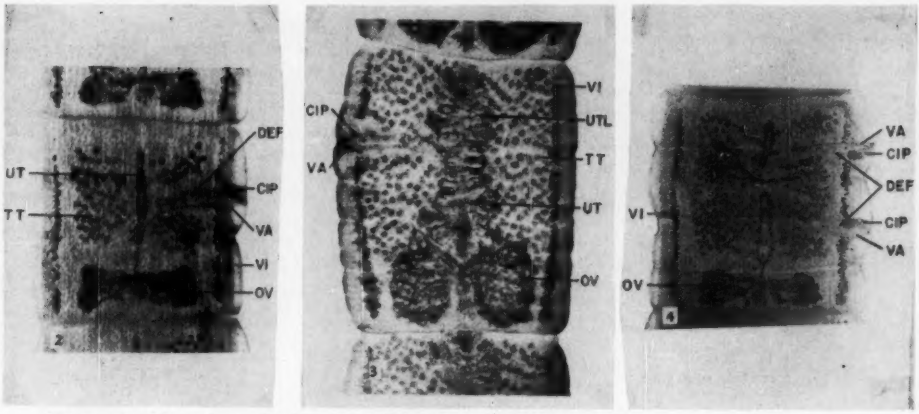

FIGURE 2. Normal proglottid of *Corallobothrium* (*M.*) *thompsoni*.
FIGURE 3. Normal proglottid of *Corallobothrium* (*M.*) *procerum*.
FIGURE 4. Proglottid of *C.* (*M.*) *thompsoni* with duplicated sex organs.

other, thus inhibiting its normal formation.

Figure 6 shows other variations in duplicated organs; there are approximately twice the number of testes, two ovaries, and two sets of genitalia. Note, however, that the ovaries are not bilaterally symmetrical. Also note that the vas deferens from the anterior cirrus organ turns anteriad rather than posteriad, whereas the vagina turns posteriad which is the normal course.

A reduction of sex organs is shown in the anterior proglottid illustrated in figure 7; that is, if the labeled septum truly separates the two segments; otherwise, the organs are duplicated. It appears to be a true septum, although it may be somewhat indistinct. The ovary is entirely absent; only the anterior tip of the uterus is present; and therefore the vagina and vas deferens end blindly. The testes are absent in the area normally occupied by the ovary and uterus. If the septum is real, there is a slight invasion of testes in the ovarian region. If this is not a true septum, then these testes belong to the posterior organ system.

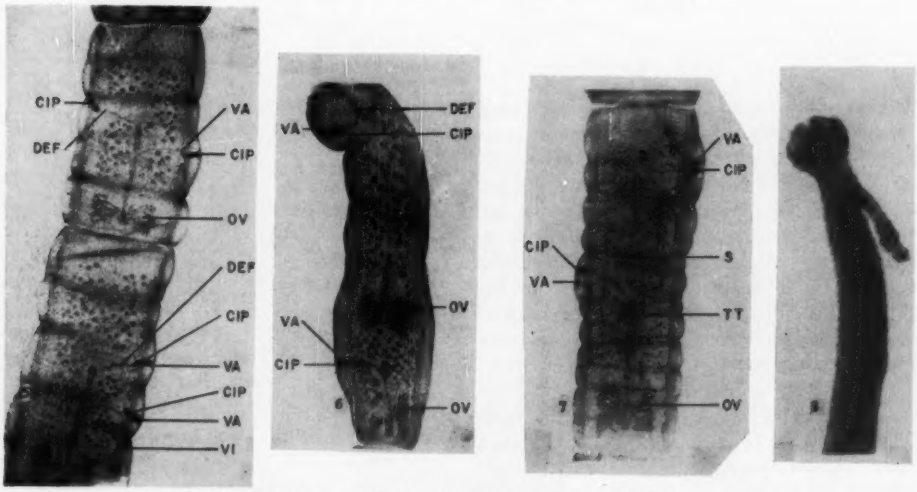

FIGURE 5. Proglottid of *C.* (*M.*) *thompsoni* showing partially duplicated genitalia.
FIGURE 6. *C.* (*M.*) *thompsoni* proglottid with duplicated sexual organs.
FIGURE 7. Proglottid (anterior) of *C.* (*M.*) *thompsoni* with reduction of sexual organs.
FIGURE 8. Immature *C.* (*M.*) *thompsoni* with two strobilae.

The immature worm of figure 8 is an example of twinning, although the secondary bud is small.

The other specimens illustrated here (figs. 8 to 10) exhibit abnormalities that may be due to mechanical injury; i.e., split or injured strobila, which show various degrees of regeneration or healing. The proglottids of figure 9 are reduced to approximately half. There was some regeneration apparently, as shown by the large knob. The worm of figure 10 appears to have regenerated portions of the right strobila but not that of the left. The posterior portion of the right strobila is also split, but the details of its anatomy are not distinct. Figure 11 is a similar specimen with complete regeneration on the right of the split (as evidenced by the median, or left vitellaria); the left side of the split remained incomplete.

FIGURE 9. C. (*M.*) *procerum* with abnormality probably due to injury.
FIGURE 10. *C.* (*M.*) *thompsoni* with bifurcated and split strobila.
FIGURE 11. *C.* (*M.*) *thompsoni* with bifurcated and split strobila.

I can offer no logical explanation of the possible cause of so many anomalous conditions in these worms. The field data do not offer any clues or facts that allow speculation.

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Abbreviations used in figures: CIP, cirrus-pouch; DEF, vas deferens; OV, ovary; S, septum; TT, testes; UT, uterus; UTL, lateral uterine pouches; VA, vagina; VI, vitellaria.

IN VITRO HATCHING OF ONCOSPHERES OF *HYMENOLEPIS DIMINUTA* (CESTODA: CYCLOPHYLLIDEA)*

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Relatively few studies have been published on successful in vitro hatching of tapeworm ova. Silverman (1954a) describes the factors affecting the hatching of ova of *Taenia saginata* and *T. pisiformis*. Jones et al (1960) studied the hatching of ova of *Taenia taeniaeformis*. To our knowledge, in vitro hatching of those cestode ova which normally hatch in an insect host has not been accomplished. In the present study we shall describe the sequence of events which lead to the hatching of ova of *Hymenolepis diminuta* and some of the factors which promote or inhibit hatching.

MATERIALS AND METHODS

Animals: The Rice Institute strain of *Hymenolepsis diminuta* carried in laboratory rats was used as a source of eggs. Beetle extracts were prepared from a Hawaiian strain of *Dermestes vulpinus* which had been kept in our laboratory for 1.5 years on a ground meat diet, and a laboratory culture of the grain beetle *Tenebrio molitor* maintained on bran flakes.

Preparation of egg concentrations: Fecal pellets of infected rats were moistened in tapwater for 15 to 20 minutes. They were then broken up with applicator sticks, the fecal material suspended in zinc sulfate and centrifuged for 1.5 minutes. The top layer of the centrifugate containing the eggs was then pipetted into clean tubes, suspended in tapwater or in distilled water (see below) and centrifuged again for 1.5 minutes. The supernatant was decanted and the eggs re-suspended. The washing procedure was repeated two more times. After the third washing, the eggs were ready for use. It was necessary to obtain eggs as free from fecal debris as possible, since the presence of much extraneous matter delayed or interfered with hatching. Eggs were used within one-half hour after being concentrated and washed; prolonged suspension in tapwater (several hours) also seemed to delay or inhibit hatching.

Tyrode's solution: Parker's Tyrode, pH range 7.2 to 7.8 (Parker, 1950), was used in all experiments unless otherwise noted. The Tyrode's solution should be freshly prepared or not more than 2 days old. If a precipitate forms, the solution should not be used. All solutions were prepared with distilled, pyrogen-free water.

Preparation of beetle extracts: Extracts of *Dermestes vulpinus* larvae or adults were prepared by macerating with a glass rod 0.5 gm of beetles in 2.5 cc Tyrode's solution and centrifuging the mixture for 20 minutes at 2000 rpm. This results in a 3-layered preparation, the bottom layer containing debris, the top layer fats, and the middle layer a relatively clear, yellowish fluid. The middle layer, which has a pH of 7.8, was used in all experiments. The extract should be used as soon as possible after preparation. The following extracts were also prepared for experimental use: (a) *Tenebrio molitor*, larvae or adults, in Tyrode's; (b) *Dermestes vulpinus* in distilled water; (c) *Dermestes* in normal (0.8 percent) saline; (d) *Dermestes* in Tyrode's which had been heated for 15 minutes at 80 C immediately after centrifugation; (e) *Dermestes* in a series of solutions containing all Tyrode's ingredients but one; for example, glucose was omitted in one, calcium chloride in the second, and magnesium chloride in the third.

Experimental procedures: Two procedures were used to accomplish hatching. The first, used in initial experiments, consisted of incubating a few drops of egg concentrate with about 7 times this amount of beetle extract at 30 C for one hour. Eggs were then placed on a microscope slide, covered with a coverslip and observed for motility of oncospheres and hatching. Hatching took place within one-half hour after incubation. When it was noted that the egg shells would not break unless the preparation was covered with a coverslip, eggs were incubated under pressure of 110 mm Hg to determine if pressure of this type would cause breakage of the shell. It was found that eggs incubated in this manner remained intact unless covered with a coverslip, indicating that contact with a solid surface or friction was necessary for breakage of the shell. Therefore, a second, more efficient procedure was devised in which the egg concentrate in a test tube was gently shaken with a few small glass beads 3 mm in diameter for 5 to 6 minutes. This resulted in breaking most of the shells; the other membranes remained unaffected. A drop of this suspension was then placed on a slide and mixed with 2 or 3 drops of extract. With this procedure hatching occurred within 20 minutes in covered as well as uncovered preparations at room temperature (25 to 27 C).

Experiments designed to determine the efficacy

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of different extracts always included controls. When extracts were prepared with distilled water, egg concentrates were also washed and suspended in distilled water. Similarly, eggs were suspended in distilled water when tested with extracts prepared with solutions other than the complete Tyrode's. *Dermestes* extract in Tyrode's was tested on eggs washed and suspended in tapwater or in distilled water.

Other procedures: For studies on egg shell structure, egg concentrates were fixed in 10 percent formalin, dehydrated in the usual manner, and embedded in paraffin. Sections were cut at 5 or 7 microns and studied under the phase microscope either unstained or stained with eosin or Gomori's trichrome. Since the higher alcohols and xylene caused many of the shells and membranes to collapse, eggs fixed in formalin were also dehydrated in a graded series of alcohol and glycerine mixtures and cleared in benzene, before embedding and sectioning. Live eggs were stained with a 1:10,000 dilution of neutral red to determine passage of the dye through the intact egg shell into the oncospohere. Adult *Dermestes vulpinus* were fed eggs of *Hymenolepis diminuta* to determine whether or not this beetle could serve as a host for the larval stages.

OBSERVATIONS

The hatching process (Plates I, II). The sequence of events which precedes the liberation of the oncosphere of *Hymenolepis diminuta* from its membranes is: (1) breaking of the egg shell and its membrane (pl. I, fig. 2); (2) activation of the oncosphere; (3) swelling and gradual disappearance of the vitelline membrane; (4) increased activity of the oncosphere which, by means of its hooks, pushes and scrapes vigorously against the thick oncosphere coat; (5) distention of the oncosphere coat which becomes thinner and opens at one end; and (6) emergence of the oncosphere from the coat and from the delicate membrane enclosing it beneath the coat.

While some oncospheres may be active before the shell is broken, activity usually is initiated after breakage of the shell. Vigorous activity of the oncosphere is apparently a prerequisite for hatching. If oncosphere activity does not continue even though the vitelline membrane has disappeared, the oncosphere coat will remain intact.

The egg shell. Sections of the egg shell show it to be composed of small blocks, some of which appear roughly hexagonal. The blocks are somewhat variable in width but fairly uniform in length. The inside surface of the shell is smooth; the outer surface is irregular because the blocks have rounded external ends beyond the point of juncture with adjacent blocks. In sections, the outlines of individual blocks are clearly visible. The boundaries between blocks appear as fine lines. It is these lines which may give a striated appearance to the egg shell when viewed intact under lower magnifications of the microscope. The egg shell and all the membranes described below are permeable to some substances, as shown by the passage of neutral red dye through intact eggs into the oncosphere.

The membranes: In addition to the egg shell proper there are four membranes enclosing the oncosphere. Beginning from the outside, the first membrane is very thin and delicate and is so closely applied to the inside of the egg shell material that it is not visible in intact eggs (pl. I, fig. 1). After the shell is empty this membrane can be seen as a wrinkled colorless structure which is partially detached from the shell (pl. I, fig. 4). The second membrane is the vitelline (pl. I, fig. 4) which at first is thicker than the shell membrane but becomes distended and thin as hatching proceeds. Between the shell membrane and the vitelline there is frequently a layer of particulate matter. Beneath the vitelline is the third membrane, often referred to as the oncosphere coat (pl. II, fig. 2). It also is relatively thick and becomes distended and thin as activity of the oncosphere continues. The fourth and last membrane, beneath the oncosphere coat can be seen clearly as soon as the embryo emerges from the oncosphere coat; it usually adheres as a thin sack to that end of the embryo which is opposite the hooks (pl. II, fig. 3).

The effect of different extracts on oncosphere motility and hatching. As already mentioned, Tyrode's extracts of adults or larvae of *Dermestes vulpinus* (pH 7.2 to 7.8) brought about hatching of oncospheres of *Hymenolepis diminuta* within 15 minutes to one-half hour after breakage of the egg shell. This procedure was used as the control with which other extracts or solutions were compared. Since it was observed that extracts prepared with old Tyrode's, containing precipitate or surface scum, did not cause hatching, different solutions were used in the preparation of beetle extracts to determine if all the components of the Tyrode's solution were essential. The possibility that one particular component of the Tyrode's solution might be essential for successful hatching was tested. Further-

more, it was noted that continuous and vigorous activity of the oncosphere was also essential to bring about hatching. The effect of exposure of the oncosphere to different extracts was therefore observed and the persistence or the absence of oncosphere motility recorded. Table I shows

TABLE I. *Effects of different extracts on oncosphere motility and hatching.*

Extract	Oncosphere activity	Vitelline membranes	Hatching	Oncosphere activity after hatching
Dermestes (larvae or adults) in Tyrode's	++	disappear	+	+
Dermestes in Tyrode's (heated for 15 min)	±	remain	–	–
Dermestes in distilled water	±	remain	–	–
Dermestes in normal saline	±	remain mostly	–	–
Dermestes in Tyrode's minus glucose	++	disappear	+	–
Dermestes in Tyrode's minus $MgCl_2$	±	disappear mostly	few or none	–
Dermestes in Tyrode's minus $CaCl_2$	+	disappear	some	–
Dermestes in Tyrode's minus $NaH_2PO_4 \cdot H_2O$	±	disappear mostly	few or none	–
Dermestes in Tyrode's minus $NaHCO_3$	±	disappear	+	±
Dermestes in isotonic glucose instead of Tyrode's	±	disappear	–	–
Tenebrio adults in Tyrode's	+	remain mostly	few or none	±
Tenebrio larvae in Tyrode's	±	remain	–	–

the results obtained with different extracts and indicates, in a general manner, the degree of oncosphere activation before hatching and its activity after hatching. It should be noted that these are subjective evaluations and that it is difficult to arrive at precise measurements of activity. As shown in table I, the omission of magnesium chloride or sodium phosphate significantly modified the effectiveness of the extract. Hatching occurred in only very few oncospheres or failed to occur even though most of the vitelline membranes had disappeared.

The omission of glucose from the Tyrode's solution did not interfere with hatching but activity of the embryos stopped after they had hatched. Substitution of isotonic glucose for Tyrode's solution did not induce hatching, although disappearance of some of the vitelline membranes did occur. Extracts prepared with *Tenebrio molitor* adults in Tyrode's (pH 7.1) stimulated good oncosphere activity but elicited hatching in a very few individuals only. The vitelline membranes remained intact in most. No hatching occurred with extract of *Tenebrio* larvae. It should be noted that distilled water was as suitable as tapwater for washing and suspension of eggs. Good oncosphere activity was observed in both types of suspensions after breakage of the egg shell, and hatching was comparable in both after the addition of the appropriate extract. The addition of Tyrode's solution only, will stimulate activity of the embryos but the membranes remain intact. Serial dilutions of *Dermestes* extracts with Tyrode's increased the time necessary for hatching. End points were not determined.

The following *Dermestes* extracts did not elicit hatching: (a) those prepared with normal saline, (pH 6.8), (b) with distilled water (pH 7.0), (c) with Tyrode's and subsequent heating for 15 minutes. Oncosphere activity was slight or absent after the addition of these extracts. The vitelline membranes eventually disappeared in a few, but oncosphere activity was not resumed.

DISCUSSION

A review of the various studies dealing with the hatching of tapeworm eggs has been published by Silverman (1954a). Silverman (1954b) has also given a detailed description of the various membranes enclosing taeniid oncospheres. He observed in addition to a layer of yolk material five membranes including the embryophore. Oncospheres of *Hymenolepis diminuta* are also surrounded by five membranes, some of which resemble those of taeniid oncospheres in their position and, superficially, in morphology. In both *Taenia* and *H. diminuta* there is a thin membrane immediately surrounding the oncosphere beneath the relatively stout oncosphere coat. The basement membrane of the embryophore in *Taenia* is in a position similar to the thin membrane which closely adheres to the inside of the blocks making up the egg shell of *H. diminuta*. The blocks, however, do not separate when exposed to the hatching solution.

From observations made on the in vitro hatching of *H. diminuta* one must assume that mechanical stress plays an important part in the hatching process. In the intermediate host,

the egg shell is probably broken as it enters the insect's digestive system. It is also possible that this breakage is facilitated by the action of digestive substances upon the thin membrane lining the shell. Thus, in our initial experiments, incubation in beetle extract with subsequent addition of a coverslip would cause breakage of the shells, while covered egg preparations without beetle extract would not show spontaneous breakage of the shells. Mechanical activity of the oncosphere probably plays an important role in the distention and bursting of the oncosphere coat and perhaps in the disruption of the membrane beneath it. When oncosphere activity does not occur these two membranes remain intact, even though the vitelline membrane may have dissolved. It is of course possible that the oncosphere, during its activity also liberates substances which act upon the coat from the inside. The vitelline membrane however, is apparently digested by a substance or substances derived from the host. Mechanical stresses such as were used to break the egg shell in our experiments never disrupted the vitelline membrane, but the vitelline can dissolve in the presence of extracts even though oncosphere activity is slight and hatching does not take place.

One of the major problems to be solved is that of the chemical and physiological factors which bring about hatching. At present, few reliable data are available on the factors which stimulate oncosphere activity. Isobe (1926) studied the effect of various drugs and of salt solutions upon the oncosphere of *H. diminuta*. He found, as did we, that solutions which stimulate initial activity do not necessarily stimulate prolonged activity. His observations were all made on intact eggs, while we were most interested in oncosphere activity after breakage of the egg shell. While oncosphere activity can be stimulated for brief periods by the addition of tapwater, distilled water, normal saline and other salt solutions either added singly or combined with beetle tissue, activity will cease within a few minutes or, if prolonged, will be weak or discontinuous. Of the solutions tested without beetle components, only Tyrode's elicited continuous and vigorous oncosphere activity for 30 minutes to one hour. Other solutions containing different ions must be tested before this problem can be resolved. The beetle extracts used in our experiments are crude and the constituents, except for the Tyrode's solution, not known. The destruction of activity of extracts by heating suggests that enzymes are responsible for hatching. Experiments to test artificial hatching solutions containing different enzymes and salts are in progress. Investigations are in progress to determine the effect of beetle extracts on hatching of different cestode eggs, particularly *Hymenolepis nana*. Eggs of *Dipylidium caninum* and of *Hymenolepis citelli* have already been hatched by the use of *Dermestes* extracts. Further experiments with these species are in progress.

SUMMARY

Oncospheres of *Hymenolepis diminuta* were hatched in vitro using extracts of adults or larvae of *Dersmestes vulpinus* in Tyrode's solution. Upon the addition of this extract, hatching occurs in 15 to 30 minutes after mechanical breakage of the egg shell. Oncosphere activity appears essential for successful hatching.

The membranes enclosing the oncosphere of *Hymenolepis diminuta* are (a) the egg shell composed of roughly hexagonal blocks, (b) a thin membrane applied to the inside of the shell, (c) the vitelline membrane, (d) the oncosphere coat, and (e) a delicate membrane enclosing the oncosphere beneath the coat.

During the process of hatching, mechanical friction is necessary for breaking of the egg shell and rupture of the underlying membrane. The vitelline membrane apparently is digested or dissolved by substances present in the host, while the oncosphere coat and membrane rupture only with prolonged oncosphere activity, whether or not the vitelline membrane has disappeared.

Extracts of *Dermestes vulpinus* prepared with distilled water or normal saline solution instead of Tyrode's do not bring about hatching. Hatching may be reduced or inhibited by extracts prepared with Tyrode's lacking certain components. Extracts of *Tenebrio molitor* in Tyrode's solution, or Tyrode's solution alone, will not elicit hatching.

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PLATE I

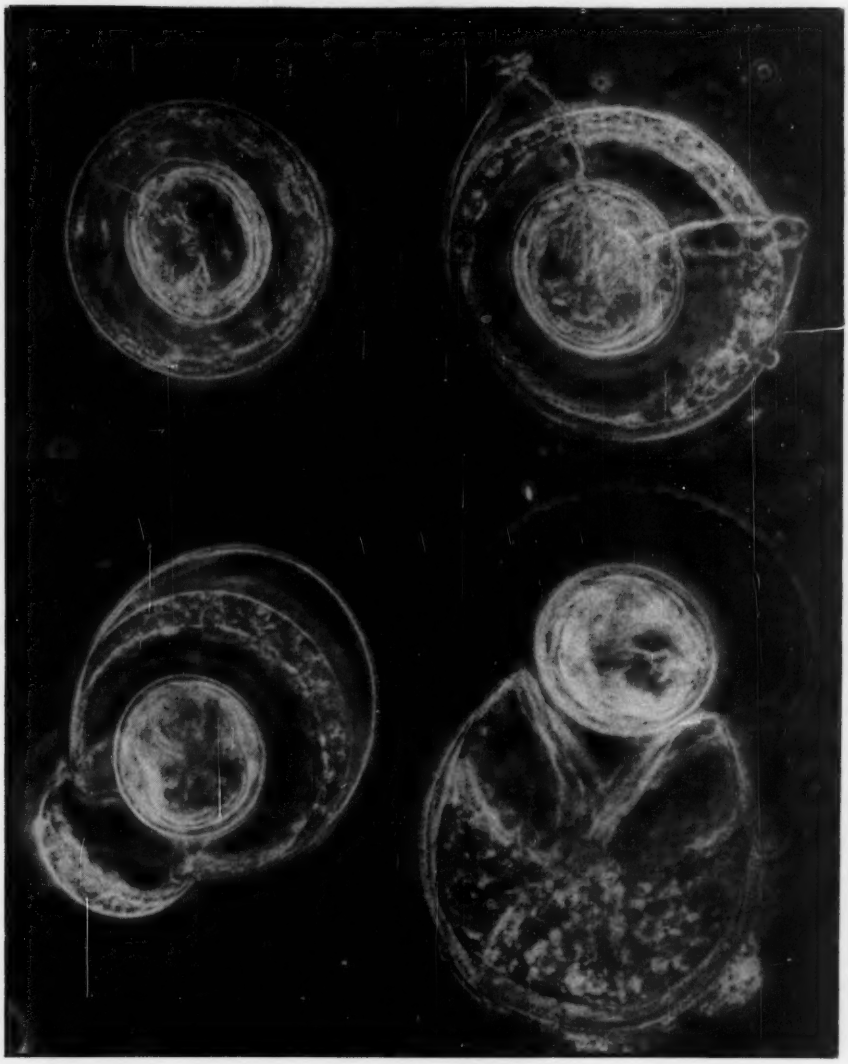
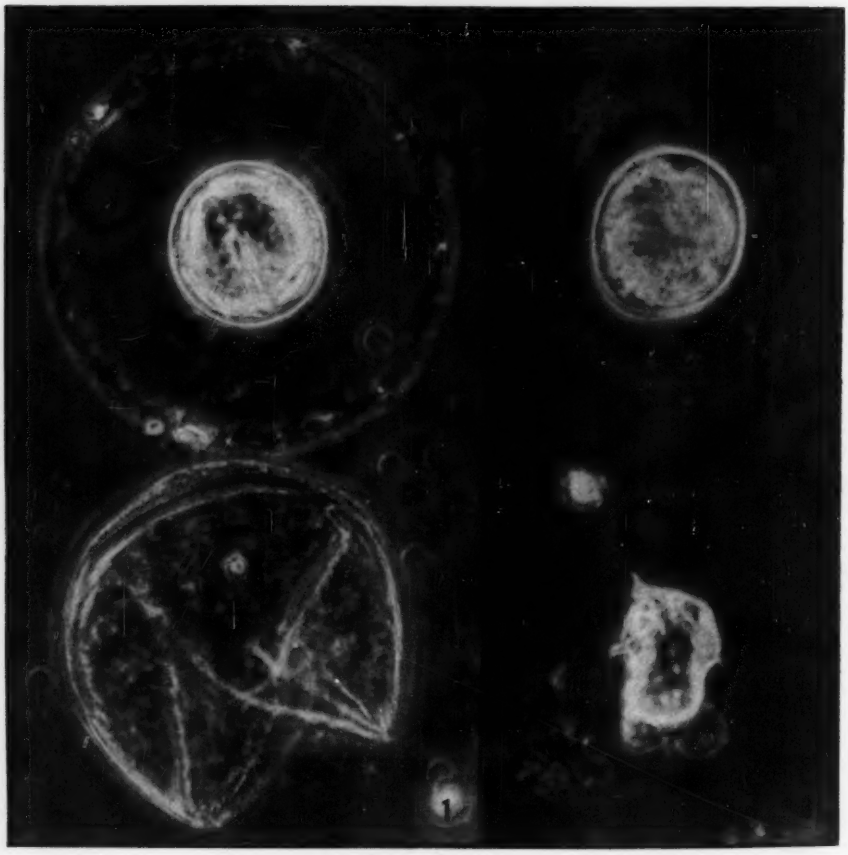


PLATE II



EXPLANATION OF PLATES

All photographs taken at the same magnification, from living material of *Hymenolepis diminuta*, under phase.

PLATE I

FIGURE 1. Intact egg.

FIGURE 2. The first step in hatching: breaking of the shell.

FIGURE 3. The oncosphere with all its coats, beginning to emerge.

FIGURE 4. The vitelline membrane separated from the shell and the shell membrane; the latter is seen as a granular mass within the shell.

PLATE II

FIGURE 1. Oncosphere with membranes completely separated from the shell below; note increase in area of vitelline membrane (pl. I, fig. 1).

FIGURE 2. Oncosphere within coat, after disappearance of vitelline membrane.

FIGURE 3. Hatched oncosphere, with oncosphere membrane at end opposite the hooks.

THE GROWTH OF THE RAT TAPEWORM, *HYMENOLEPIS DIMINUTA*, DURING THE FIRST FIVE DAYS IN THE FINAL HOST*

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During the past 15 years many studies have been made on various metabolic and physiological processes in the adult rat tapeworm, *Hymenolepis diminuta* (Rudolphi, 1819). As a result of these studies our knowledge of this species and of tapeworms in general has been increasingly broadened.

Chandler (1939) contributed experimental data on the location of this tapeworm within the intestine of the rat host, and Chandler (l.c.), Hager (1941), Read (1951), and Roberts (1960) have studied the effects of increasing the size of the population within the host, the so-called crowding effect.

At present our knowledge of early growth and development of *H. diminuta* is quite fragmentary. The present study was undertaken, therefore, to learn more about early infection in the rat. Basically, the study consisted of an investigation of excystation, growth, and location of worms during the first 5 days, and a morphological study of mature and excysting cysticercoids and early changes in young worms.

MATERIALS AND METHODS

A total of 36 young, sexually mature Wistar strain rats was used during the course of these experiments. All rats were fed a standard laboratory rat chow, and all remained in excellent health as demonstrated by weight increases, alertness, and sleek and robust appearance.

The strain of *Hymenolepis diminuta* and its maintenance in the laboratory are the same as given in earlier papers (*cf* Goodchild, 1958). Infected beetles were kept at 27 C during cysticercoid development and the majority of experiments utilized cysticercoids 21 to 33 days old, but some were used when only 12 days old and others when 54 days old. The following technique was employed to infect rats and to recover tapeworms. Several beetles were teased and cysticercoids liberated into 0.85 percent NaCl. The required number of larvae was sucked up into a small-bored polyethylene tube attached to a glass syringe and the tube was inserted through the esophagus into the stomach of a lightly etherized rat and the cysticercoids expelled. All rats were finally killed by an overdose of ether and the small intestine (and stomach in some cases) was removed from the body within a few minutes after death. The intestine was promptly submerged in normal saline, was transected into fourths or sixths, and examined for worms.

Numerous techniques to recover sufficiently large numbers of tapeworms were tried unsuccessfully in early phases of this work. Very young worms, especially during the first 48 hours, are small and difficult to distinguish from villi of the intestinal mucosa, being of the same approximate size, shape, and color. First attempts at recovery consisted of cutting the intestine along its entire length, pinning it open in a dissecting tray, and searching each region with a dissecting microscope. This technique was laborious, usually requiring 4 to 6 hours per rat, and so inefficient that it was discontinued. In efforts to alleviate the difficulties of recovery, cysticercoids were stained with vital dyes before being fed to rats. Neutral red (1:20,000) and Nile blue sulfate (1:50,000) stained the cysticercoids, but after 24 hours in the rat the color had disappeared.

Finally, a satisfactory method of recovery was devised. Following removal and transection of the intestine, each section was opened longitudinally and pinned under normal saline. The entire mucosal layer from each section was scraped with a dull scalpel and transferred to separate Petri dishes containing normal saline. The scrapings were further torn with dissecting needles until only small fragments remained. Dishes were placed about 12 in. under a 45-watt lamp in a reflector. After 2 to 3 hours each container was examined with a dissecting microscope and young worms crawling on the bottom were recovered. After 72 hours worms are large enough to see in situ and the scraping technique was not required.

Specimens were relaxed in cold, normal saline and fixed in Bouin's, Heidenhain's Susa, or Helly's fluids. The following stains were used: Heidenhain's iron alum hematoxylin, Delafield's, Ehrlich's and Harris' hematoxylin, borax carmine, alum cochineal, Semichon's carmine, a combination of alum cochineal, Delafield's hematoxylin, and water (3:1:25), Mallory's triple stain, and Masson's trichrome stain. In addition, dilute neutral red and Nile blue sulfate were used in limited in vivo

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observations of cysticercoids and young worms.

Two measurements, length and area, were used for comparing sizes of populations. The mean length of each age group is given since this is the usual criterion employed by other workers. In addition, comparisons of areas were employed. Areas were determined by two methods, a computation based upon length times average width, and an index determined by a paper weight equivalent. The former method proved to be as reliable as the paper weight index (as will be discussed below) and was, accordingly, the method adopted. Lengths and widths of stained worms were obtained from projected images. The scale of a stage micrometer was projected at the same magnification to give actual sizes. To obtain the paper weight equivalent, outlines of a few worms were traced on sheets of paper, were cut and weighed to obtain relative indices of area.

A total of 588 tapeworms was prepared and studied, including 52 specimens sectioned in paraffin (by standard methods) and 536 whole mounts. Well over 200 cysticercoids were also prepared and studied. Included in the specimens examined were mature encysted and excysted cysticercoids, and young worms 4, 5, and 10 hours, and 1, 2, 3, 4, and 5 days old.

OBSERVATIONS AND RESULTS

Growth of Worms during the First Five Days in the Rat

Prior to the use of the scraping method for recovering young tapeworms only 0.4 percent of 1,500 cysticercoids given to 10 rats were later reclaimed as worms. By using the scraping method 50.5 percent of the next 1,267 given to 20 rats were recovered as worms after 48 hours.

To study the process of excystment 50 cysticercoids were administered to a rat once an hour for 3 hours, and 1 hour later it was sacrificed. The stomach and small intestine were removed, the contents scraped into Petri dishes and examined. Three distinct populations were recovered, two in the stomach and one in the duodenum. One group from the stomach could still be recognized as cysticercoids, but with much of the outer cell layers digested away; the second consisted of only the central portion of a cysticercoid, i.e., the scolex within the thick, innermost membrane (fig. 7). These two populations were apparently 2 and 3 hours old, respectively. No excysted worms were recovered from the stomach. Young worms (similar to figs. 8 and 9), considered to be 4 hours old, were found in duodenal scrapings. Cysticercoids were not present in the duodenum, nor were specimens observed in the process of excysting in scrapings from either stomach or intestine. Failure to observe excystment in the rat is not surprising since observations we have made in vitro suggest that after proper priming actual emergence in vivo may take place within a few minutes.

The following experiment was designed to discover what effect, if any, the age of the cysticercoid might have on the rapidity with which it might excyst and on the initial size of the worm. One female rat was given about 50 larvae 12 days old, and another female received the same number of 54-day-old larvae; both hosts were killed after 2 hours. All larvae were within the stomach and digestion had reduced the younger larvae to hardly more than scoleces, whereas only the outer-most layer of cells had begun to disintegrate in the older specimens. Five other female rats were each given similar doses of cysticercoids varying from 22 to 33 days in age and were killed 48 hours after infection. The group of worms with the largest mean area, and the population with the smallest, came from cysticercoids in which there was but 1 day's difference in age (32 and 33 days old); the second largest mean area was found, moreover, in worms coming from the youngest cysticercoids (22 days).

Most of the rats used throughout these experiments were infected with large numbers of cysticercoids (100 or more) to reduce the number of hosts needed, yet provide sufficient specimens for study. Chandler (1939) reported lack of the crowding effect before day 11; however, he gave fewer cysticercoids than used here. By introducing many cysticercoids it seemed probable that the crowding effect might be evoked at an earlier age. To test this possibility three pairs of female rats were used. One member of each pair was given a much larger dose of cysticercoids of approximately the same age. Each pair was sacrificed at the same time, after 48 or 72 hours, and comparisons were made between worms recovered from each rat. As shown in table I, which summarizes these comparisons, a wide variation occurred between worms 48 hours old. In one pair, the worms from the larger infection (100) averaged more than 3 times the area of those from the smaller (40), and the ranges did not overlap. However, in the second pair, worms from the smaller infection (20) averaged more than 4 times the area of those from the larger (40), and ranges, again, did not overlap. Areas varied less in worms 72

TABLE I. *Effect of the size of the dose on the growth rate of* H. diminuta.

Age of worm (hrs.)	No. of cysticercoids given	Age of cysticercoids (days)	No. worms recovered	Mean area of worms ($\times 1000\mu^2$)	Standard error of mean ($\times 1000\mu^2$)	Area range ($\times 1000\mu^2$)
48	100	33	49	71.5	± 1.74	47–95
48	40	32	10	23.6	± 1.91	17–31
48	40	22	11	12.5	± 0.82	10–16
48	20	22	9	54.5	± 6.00	41–77
72	100	33	66	209.2	± 7.35	90–310
72	10	33	5	192.5	± 28.35	130–280

hours old; those from the infection of 100 cysticercoids had somewhat larger mean areas than those from an infection of only 10 cysticercoids.

Lengths of worms in six age groups were measured and areas calculated to determine the rate of growth during the first 5 days. The six groups included 150 specimens recovered at 5, 24, 48, 72, 96, and 120 hours post-infections. on 401 worms in the same six age groups. The worms showed area increases of 1.3× the first day, 4× the second day, 4.2× the third and fourth days, and 2.2× the fifth day. Figure 1 depicts these results plotted on a semi-logarithmic scale; it will be noted that the resulting curves are essentially exponential in form during part of the time. The general exponential

TABLE II. *Growth rate, in length, of* H. diminuta *during the first five days in the rat host (including the data reported by Chandler, 1939).*

Size of population	Age of worms (hrs.)	Mean Length (microns)	Range	Daily increase
15	5	160.3 ± 5.5	135– 215	—
20	24	204.5 ± 6.7	170– 280	1.3
58	48	545.0 ± 1.7	250– 756	2.6
24	72	1,860.0 ± 85.6	1,050– 2,475	3.4
19	76	5,632.0 ± 263.4	3,600– 8,000	3.0
14	120	14,686.0 ± 1,079.7	8,800–20,800	2.6
Chandler, A. C.		(Average)		
—	24	270	240– 320	—
—	48	330	210– 380	1.2
—	72	1,150	790– 1,500	3
—	96	3,760	2,000– 6,700	3
—	120	14,000	8,000–20,000	4

Table II summarizes the findings and also includes a part of Chandler's (1939) data to allow comparisons between the two studies based upon length. In this work it was determined that length increased by 1.3× the first day, 2.6× the second day, 3.4× the third day, 3× the fourth day, and 2.6× on the fifth day. In addition, calculations for area, given in table III, were made

TABLE III. *Growth rate, in area, of* H. diminuta *during the first five days in the rat host.*

Total worms	Age of worms (hrs.)	Area of the worms (mean) ($\times 1000\mu^2$)	Range of the area ($\times 1000\mu^2$)
45	510	9.4 ± 0.27	6– 15.0
49	24	12.6 ± 0.96	8– 18.5
131	48	51.1 ± 0.89	10– 95
143	72	213.7 ± 4.16	90– 310
19	96	900.0 ± 71.30	300–1,800
14	120	2 010.0 ± 202.70	1,300–3,500

growth formula expressed by $X_t = X_0 e^{K_1 t}$, where X_t is the final value (in this study for length or area), X_0 the original value, e the irrational number 2.71828, K_1 the instantaneous or geometric growth rate, and t the time elapsed, has been solved for data collected in this study. For any time interval beyond 24 hours and up to 5 days, K_1 for length is equal to approximately 1.10, but is equal to only 0.24 during the first day; for area, K_1 is about 1.41 between day 1 and 4, is 0.29 for day 1, and 0.80 for day 5; these values are depicted in figure 2. It must be remembered that growth during the first day is limited to about 20 hours, since about 4 hours may be required for excystment.

Previous workers have shown that sex of the host rat influences the size and egg productivity of *H. diminuta*. In the present work, fe-

males were used almost exclusively, but an attempt was made to detect differences in growth rates of young tapeworms in the two sexes. Three pairs of rats, consisting of one male and one female each, were given the same number of 28-day-old cysticercoids. Pairs were sacrificed at 24-hour intervals; table IV summarizes the findings. After 24 hours there was little difference in mean areas of worms from either sex, but after 48 and 72 hours mean areas of worms from males were slightly larger. However, the percentage of size difference between the two groups was somewhat less at 72 hours than at 48 hours.

TABLE IV. *Effect of the sex of the host on the growth rate of* H. diminuta.

Age of worm (hrs.)	No. of cysticercoids given	Age of cysticercoids (days)	No. of worms recovered	Sex of host	Mean area of worms ($\times 1000\mu^2$)	Standard error of the mean ($\times 1000\mu^2$)	Area range ($\times 1000\mu^2$)
24	100	28	31	F	12.8	± 0.32	10– 17.5
24	100	28	36	M	12.5	± 1.50	8– 18.5
48	100	28	33	F	40.5	± 2.21	14– 65
48	100	28	53	M	45.5	± 0.65	37– 55
72	100	28	72	F	211.3	± 5.42	88–327
72	100	28	86	M	221.5	± 4.12	130–280

Position of Worms in the Rat during the First Five Days

To locate worms within the intestine during the first 5 days the statistics given in table V were gathered from 11 rats. The scraping method was employed to collect all specimens except the 4 and 5-day-old ones. The intestines of eight rats (females) were transected into fourths; the data given in the table include the number and percentage of worms recovered from each fraction. As shown in table V, 74 percent of all 4-day worms, and 68 percent of all 5-day worms, were found in tight masses about 25 to 28 cm behind the stomach. Measurements showed that these worms were clumped at the exact center of the anterior half of the intestine. To determine whether younger worms in male rats also congregated in this narrow zone, intestines from three male rats were sectioned into sixths. From table V it may be seen that the second sixth, as before, had the greatest percentage of worms.

Morphological Changes in the Cysticercoid and in Young Worms during the First Five Days in the Rat

Mature cysticercoids possess a narrow canal which leads directly from the area of the scolex up through the anterior tip of the larva where it becomes somewhat widened at an anterior opening (figs. 1, 2, 10). In cross section the

TABLE V. *Distribution of* H. diminuta *in the small intestine of the host rat during the first five days.*

Age of worm (hrs.)	Sex of rat	Number and (percentage) of worms found in the quarters of the intestine			
		1st	2d	3d	4th
4	Female	22 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
5	"	27 (81.1)	6 (18.2)	0 (0.0)	0 (0.0)
24	"	3 (3.3)	28 (96.7)	0 (0.0)	0 (0.0)
48	"	4 (12.1)	26 (78.8)	3 (9.1)	0 (0.0)
48	"	6 (12.2)	40 (81.5)	3 (6.3)	0 (0.0)
72	"	3 (5.0)	60 (90.0)	3 (5.0)	0 (0.0)
96	"	4 (8.7)	37* (80.5)	3 (6.5)	2 (4.3)
120	"	0 (0.0)	53** (100.0)	0 (0.0)	0 (0.0)

Age of worm (hrs.)	Sex of rat	Number and (percentage) of worms found in the sixths of the intestine			
		1st	2d	3d	4th, 5th, 6th
24	Male	0 (0.0)	21 (58.4)	15 (41.6)	No data
48	"	16 (30.2)	31 (58.5)	6 (11.3)	" "
72	"	10 (11.6)	75 (87.2)	1 (1.2)	" "

* 34 of 37 (92 pct.) were found from 25 to 27 cm behind the stomach.

** 36 of 53 (68 pct.) were found from 25 to 28 cm behind the stomach.

TABLE VI. *Comparison of three techniques employed to determine the size of* H. diminuta.

Series	Age of worms (hrs.)	Length (mean) (microns)	Average width (microns)	Area (L × W) (mean) (× 1000μ²)	Paper weight (mean) (mg)
K	48	456.7	88.6	40.5	32.3
M	48	616.8	115.9	71.5	56.5

$$\frac{\text{Area of K}}{\text{Area of M}} = \frac{40.5}{71.5} = 56.6 \text{ pct.}$$

$$\frac{\text{Paper weight of K}}{\text{Paper weight of M}} = \frac{32.3}{56.5} = 57.1 \text{ pct.}$$

anterior end of the cysticercoid reveals a biradial symmetry (figs. 10–15). The anterior canal, a short distance behind the anterior opening, becomes compressed apparently by forward growth of two chambers confluent with the large cavity within the cysticercoid (figs. 11, 12, 13, 14). In fact, the anterior canal becomes nearly occluded centrally, but is still visible laterally (figs. 11, 12, 13). The worm is attached to the anterior canal by a cuticulated, annular-like zone (figs. 6, 13, 14, 15). In sections cut from anterior to posterior the outer layer of the young worm (*cf* below, and Voge, 1960a, b) is revealed (figs. 16, 17) before the main body of the scolex (figs. 18). The scolex within the cavity of the cysticercoid is cuticulated from the anterior end to a point somewhat below the suckers. Posterior to the suckers the cuticula is sharply reflected upon itself and invests the scolex as a second layer (figs. 1, 2, 6, 7, 18). Darkly staining cells fill the scolex from the anterior end to the point of reflection; here, the cells form a loose and irregular layer adherent to the cuticula on one side, but are exposed to the cysticercoid cavity on the other. The cysticercoid thus contains a young worm which is approximately half inverted. During excystation, which we have observed repeatedly in vitro, the time elapsed from initial agitation of the young worm to complete escape from the larval envelopes may be only 3 minutes. As the scolex probes into the anterior canal the reflected posterior cuticula and cell layer are forced to snap into a reversed spatial relationship (figs. 2, 3, 4, 5). The reflected cell layer of the scolex now becomes located internally behind the suckers and forms the boundary of a large, central, seemingly non-functional cavity which opens posteriorly by a pore, the former anterior attachment of the worm to the larval envelopes (figs. 8, 9). It is at this point that the excretory canals open and this method of semi-eversion may explain the occurrence of a common excretory pore in this tapeworm prior to loss of the terminal proglottid. By the end of 24 hours in the rat the posterior cavity has been occluded by ingrowth of parenchymatous cells from the periphery.

When the tapeworm emerges from the cysticercoid it is more than a simple scolex; it is a tiny worm (figs. 8, 9). At least two branches of the excretory canals, the dorsal pair, are developed and apparently functioning before the worm emerges. In the encysted state these canals

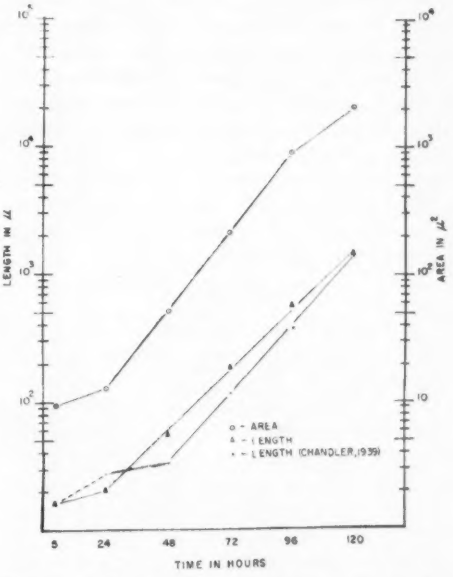

FIGURE 1. Increases in length and area of *H. diminuta* during first 5 days in the rat host, including a comparison of increases in length found in the present study with those found by Chandler (1939). All values are plotted on a semi-logarithmic scale.

can be traced from the scolex down the inner sides of the cuticula and into the tissue which is reflected over the anterior end of the scolex. Flame cells are functioning, at least cilia are active, in the tissues of the scolex, including the reflected layer; none were visible in the envelopes or tail of the cysticercoid. Shortly after the cuticula forms the continuous covering around the young worm, and after the occlusion of the posterior cavity, the canals migrate toward the center of the strobila and remain close

together until segmentation and differentiation of the reproductive *Anlagen* force them farther apart 4 days later. Whether the ventral pair of canals is present before excystation is not known, but by the end of 24 hours they are visible and presumably are functioning.

Before and after occlusion of the posterior cavity a distinct gradient of cells exists throughout the young worm (fig. 9). Within the scolex there is a remarkable sparsity of cells. Scattered loosely among the permanent organs of the scolex are mesenchyme-like cells. Behind the scolex two major types of cells occur in the strobila of 24 to 48 hour worms. Beneath the cuticula is a layer of rounded cells which tend to mass posteriorly into a layer several cells thick. The second cell type is confined to the medulla and is similar in position to the adult parenchyma. The cells here are generally elongate, spindle shaped, and lie with their long axes perpendicular to that of the worm. By the third day the cortical cells, formerly rounded, now become elongated and stand perpendicular to the cuticula which they undoubtedly secrete.

DISCUSSION

A study of growth and maturation of tapeworms is complex since many variables operate to influence the overall patterns. Chandler (1939) and Read (1951) found an inverse relationship between lengths of adult *Hymenolepis diminuta* and the size of the populations, the so-called crowding effect. In this study, and in a related study reported in abstract by Roberts (1960), no crowding effect was revealed even with 100 or more worms; by 5 days, however, worms may still be too small to compete intensively for space and nutrients as they might later.

Young worms in male or female hosts were found in this study to be approximately equivalent in length. It was true that mean areas of worms in male hosts were larger after 48 and 72 hours than those in female hosts; however, percentage differences were less after 72 hours than after 48 hours. Although the means of pairs of population differed somewhat, in each case there was no statistically significant difference between them (probability of significance for *t*-test was greater than 0.05).

In the present study it has been shown that the age of the cysticercoid has little, if any, effect upon the subsequent rate of early development of the worm. Voge and Heyneman (1957) stated that growth of the scolex within the cysticercoid ceased after the larva had reached maturity within the beetle, and Rothman (1959) maintained that younger cysticercoids excysted more rapidly than older ones. Both of these findings are confirmed in our study. Older cysticercoids do, in fact, take longer to excyst, but after 48 hours no differences could be detected between worms from older or younger cysticercoids.

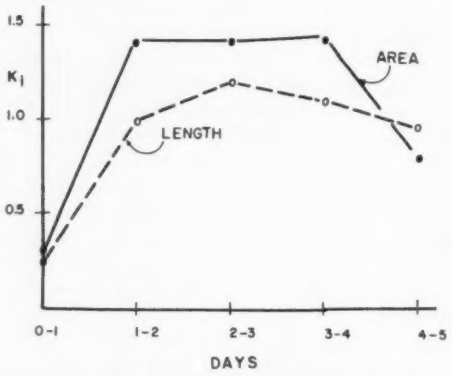

FIGURE 2. Geometric growth rate values (K_1) for length and area of *H. diminuta* during first 5 days in the rat.

The present work does not confirm earlier results regarding the place of initial establishment of worms in the rat's intestine. Chandler (1939) stated, ". . . the worms make their initial attachment far back in the intestine, 80 percent of them 40 centimeters or more behind the stomach. They subsequently move forward, between the 7th and 10th days, to their ultimate anterior position." This report, which is based upon smaller populations, differs from our results (*cf* table V). In the first few hours after excysting most specimens were found to be in the first quarter of the intestine. During the first 24 hours 97 percent moved posteriorly into the second quarter, and between the 48th and 96th hours only a few specimens were recovered from posterior regions. By the end of day 5 all specimens were found in the second quarter only. In 4-day-old worms 74 percent were concentrated in an area 25 to 27 cm behind the stomach, and in 5-day-old worms 68 percent were still concentrated in an area 25 to 28 cm behind the stomach. This finding, too, is in disagreement with Chandler (*l.c.*) who stated

that worms were usually found in groups of 2 or 4 at intervals of 6 to 15 cm.

The absolute and/or relative size of tapeworms has been measured in various ways. Voge (1952) discussed the criteria used by several investigators to obtain linear measurements of such structures as the rostellum, the scolex, etc.; these criteria will not be further discussed here. Read (1951) described the effect of crowding upon the size of adult worms and evaluated methods employed by investigators to determine the actual decrease in size; for example, Chandler (1939) and Hager (1941) used length, Reid (1942), with *Raillietina cesticillus*, compared weights, and Wardle and Green (1941), with *Diphyllobothrium latum*, used weight: length as an average cross sectional area. Read (*l.c.*) used the area ($= \text{weight}^{2/3}$) divided by the weight to obtain an index of the "relative surface area." With the exception of a part of Chandler's work all of these studies have utilized adult worms.

In the present investigation it became necessary to decide upon the most valid criterion for comparative measurements. Obviously, weighing seemed impractical because the several hundred minute specimens were to be utilized for histological and cytological studies, and could not be subjected to possible damage. Because contraction and elongation were difficult to control, it was considered unwise to use length as the sole measurement. Also, mathematical determination of area seemed to present theoretical hazards since worms were often tapered, being wider posteriorly than anteriorly. An area index based upon a paper-weight-equivalent seemed to offer promise of reliability; as mentioned above, outlines of permanently prepared specimens were projected and traced on paper, were cut and weighed to obtain a comparative index of area for each population studied.

A preliminary trial to test reliability of these three types of measurements was run on two populations of worms, K and M, recovered after 48 hours from female rats (table VI). The ratio of areas (length × width) of series K to series M differed by only 0.5 percent from the ratio of the paper weight equivalent areas. Computation of areas, therefore, appeared as accurate as area indices determined by the more laborious paper weight equivalence; the latter method, after this preliminary trial, was discontinued and areas given throughout the paper were products of lengths times average widths. In this small series, length measurements alone would have been sufficiently reliable for determining difference in growth since they were, in fact, less divergent than the areas; for example, areas of K were about 57 percent of those of M, whereas lengths of K were 73.8 percent of lengths of M worms. Closer approximation of lengths than areas in the two groups indicates differences in widths; in fact, K worms were only 55.6 percent as wide as M ones. It is regrettable that weights were not determined for these two populations of 48-hour worms, since it seems apparent that the M series should have been heavier than the K group.

Attention has previously been drawn to figure 1 and table II which list lengths given by Chandler (1939) and those determined in the present investigation. Basically the two growth patterns are similar and they may be statistically equivalent. Chandler commenced his study with 24-hour-old specimens and this initial datum is shown in the figure. Since the strain of *H. diminuta* which we used was obtained originally from Rice University, it is considered likely that sizes of newly excysted worms in the two studies would be essentially similar; hence, the common origin at 5 hours on the figure, and the assumed change in length between 5 and 24 hours (given as the dotted line segment) of Chandler's specimens.

In our study the rate of growth reached a maximum on the third day during which the length was more than tripled (3.4 times that of the second day). On the fifth day worms grew only 2.6 times longer than their length of the fourth day. In Chandler's data, however, the fifth day represents the most productive of the first 5 days with a total elongation 4 times that of the length of the fourth day.

It was found in this study that length and area each increased by only 1.3 × during the first day and this was considerably less than that recorded for each of the next four days. However, it should be recalled that the new worm is essentially a hollow sac extending behind the scolex and that the cavity disappears during the initial 24 hours. Rapid cell proliferation inward to occlude this cavity may prevent an appreciable increase during the first 24 hours. Only after the cavity has been filled with cells does the worm increase rapidly in length and width. The geometric growth rate, K_1, for area was

remarkably constant in *H. diminuta* between days 1 and 4, but was depressed between days 4 and 5. Most rapid increases in area were thus found between excystation and day 4. Whether declines occurred in area growth rates beyond day 5 is not known because the study terminated on that day. Maximum geometric increases in length occurred up to day 3 with slight decreases evident during the next 2 days. Schiller (1959) provided data for *H. nana* during equivalent periods of time in two hosts, the albino mouse and the gray squirrel. Computations revealed that in the gray squirrel, between days 3 and 5, K_1 values ($K_1 = 0.97$ for length) were essentially equivalent to those found in the present study; in the mouse, however, geometric increases in length from days 3 to 9 (K_1 varied between 0.43 to 0.53) were much less than those he found in the squirrel, or those found in the present study. Schiller's figures suggest that in the mouse the growth rate may be relatively constant from day 3 to day 9 (these are the ranges used in the K_1 computations), but in the squirrel a decrease in rate occurred between day 5 and 7 ($K_1 = 0.07$), but the growth rate increased—with a different slope ($K_1 = 0.26$)—between days 7 and 11. In abstract form, Roberts (1960) has presented data which corroborates many of the findings of our study. He, too, found a lag in growth during the first day which was followed by an exponential phase during the next 5 to 6 days. Furthermore, regardless of population density, growth curves of all worms were similar until retardation in growth was reached at 15 to 17 days.

SUMMARY

A scraping method for recovering young *Hymenolepis diminuta* from the small intestine of Wistar strain rats has been described and evaluated. This method is particularly useful during the first 72 hours after infection.

During the first 3 days worms recovered from male and female hosts showed no significant differences in size, nor was a crowding effect manifest even with massive infections (100 or more cysticercoids).

Larval envelopes of cysticercoids 54 days old resisted gastric digestion better than did those on larvae only 12 days old. Young worms emerge from the cysticercoid between 3 and 4 hours post-infection apparently in the small intestine after priming in the stomach. Descriptions are given of the process of excystation and of histological details of the cysticercoids and young worms.

Comparisons are made between localization of worms during the first 5 days with the findings of Chandler (1939). The majority of young *H. diminuta* tends to cluster midway down the anterior half of the small intestine; on the fifth day 68 percent were congregated in a 3 cm length at this midpoint.

Data are presented showing that the rate of growth of *H. diminuta* during most of the first 5 days is exponential in character. Increases in length and area were studied and comparisons were made of their relative reliability as indices of growth.

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EXPLANATION OF PLATES

All figures are photographic enlargements from 35 mm negatives.

PLATE I

FIGURE 1. Longitudinal section of anterior region of mature cysticercoid. The cells at end of arrow are the same cells as at end of arrow in figure 9.

FIGURE 2. Whole mount of anterior region of mature cysticercoid. Lines and numerals on this figure refer to approximate levels of transection of corresponding figures on Plate II.

FIGURE 3. Beginning of excystation of worm from cysticercoid. Note probing of rostellum into anterior canal, flattening and elongation of suckers, and sequence of reversal of relationship of post-sucker layers of young worm (at end of arrow).

FIGURE 4. Young worm nearly one-half excysted.

FIGURE 5. Young worm in process of excystation showing posterior cavity behind zone of suckers.

FIGURE 6. Longitudinal section of cysticercoid showing (at end of arrow) annulated and cuticulated zone of attachment of worm to larval envelopes.

FIGURE 7. Two unexcysted cysticercoids after 3 hours in a rat's stomach. Note total digestion of outer cell layers of larvae.

FIGURE 8. Young worm showing large posterior cavity approximately 6 hours after excystation in vivo.

FIGURE 9. Longitudinal section (2 microns) of a worm less than 2 hours after excystation. Note large posterior cavity, sparsity of cells in anterior region, and beginning of migration of peripheral cells to occlude cavity. The cells at end of arrow are the same cells as at end of arrow in figure 1.

PLATE II

Skip serial cross sections of anterior portion of mature cysticercoid showing biradial symmetry. Level of transections are indicated by corresponding numerals on figure 2; intermediate figures are cut from intermediate regions. Sizes of figures may be determined approximately from figure 18, the actual largest outer diameter of which is 0.248 mm. Main features are indicated by arrows on the figures.

FIGURE 10. Showing central canal which is lined with the inturned outer fibrous layer.

FIGURE 11. Central canal compressed by the two anterior extensions of the main cysticercoid cavity.

FIGURE 12. Central canal nearly occluded in the middle, but persisting laterally.

FIGURE 13. Central canal near junction with outer layer of young worm, showing ring-like connection. Laterally, on the left, the anterior canal is still present.

FIGURE 14. Ring-like junction of worm and larval envelopes show centrally. Cell masses above and below isthmus are in anterior portion of main cysticercoid cavity. They are outside tissues of worm and remain within cysticercoid after excystation.

FIGURE 15. Showing junction of worm and larval envelopes. Cell masses in cavity are tangential cuts of outer cell layer of young worm.

FIGURE 16. Ring cell mass is subtangential cut of outer layer of young worm. Region of scolex not yet transected.

FIGURE 17. As in figure above, but transected slightly more posteriorly.

FIGURE 18. Cross section through suckers. Note, in scolex area, inner and outer (at end of arrow) layers of cuticula and layer one or two cells thick outside outer cuticula. These outer cells by inversion become located behind scolex area during excystation.

PLATE I

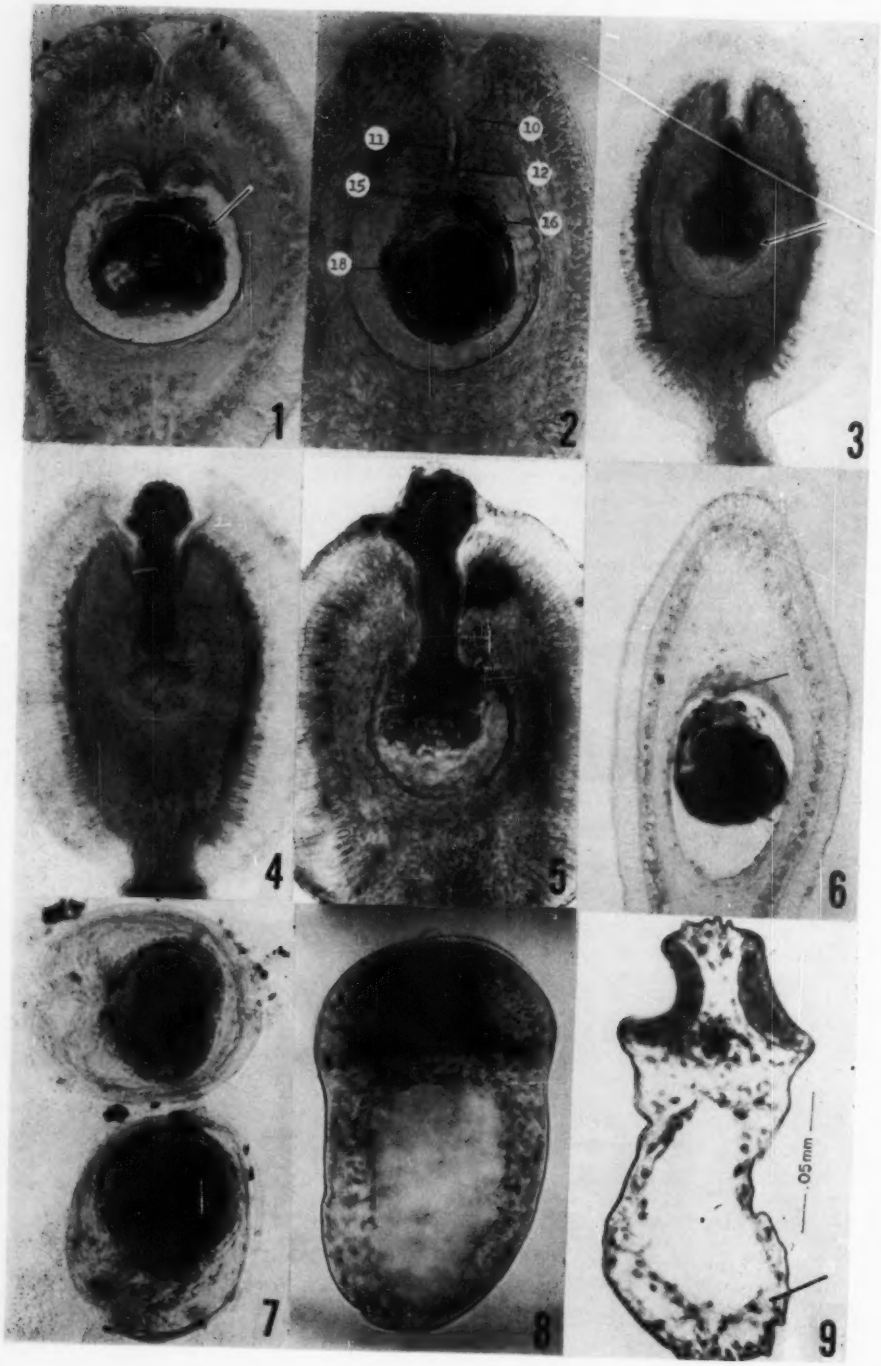
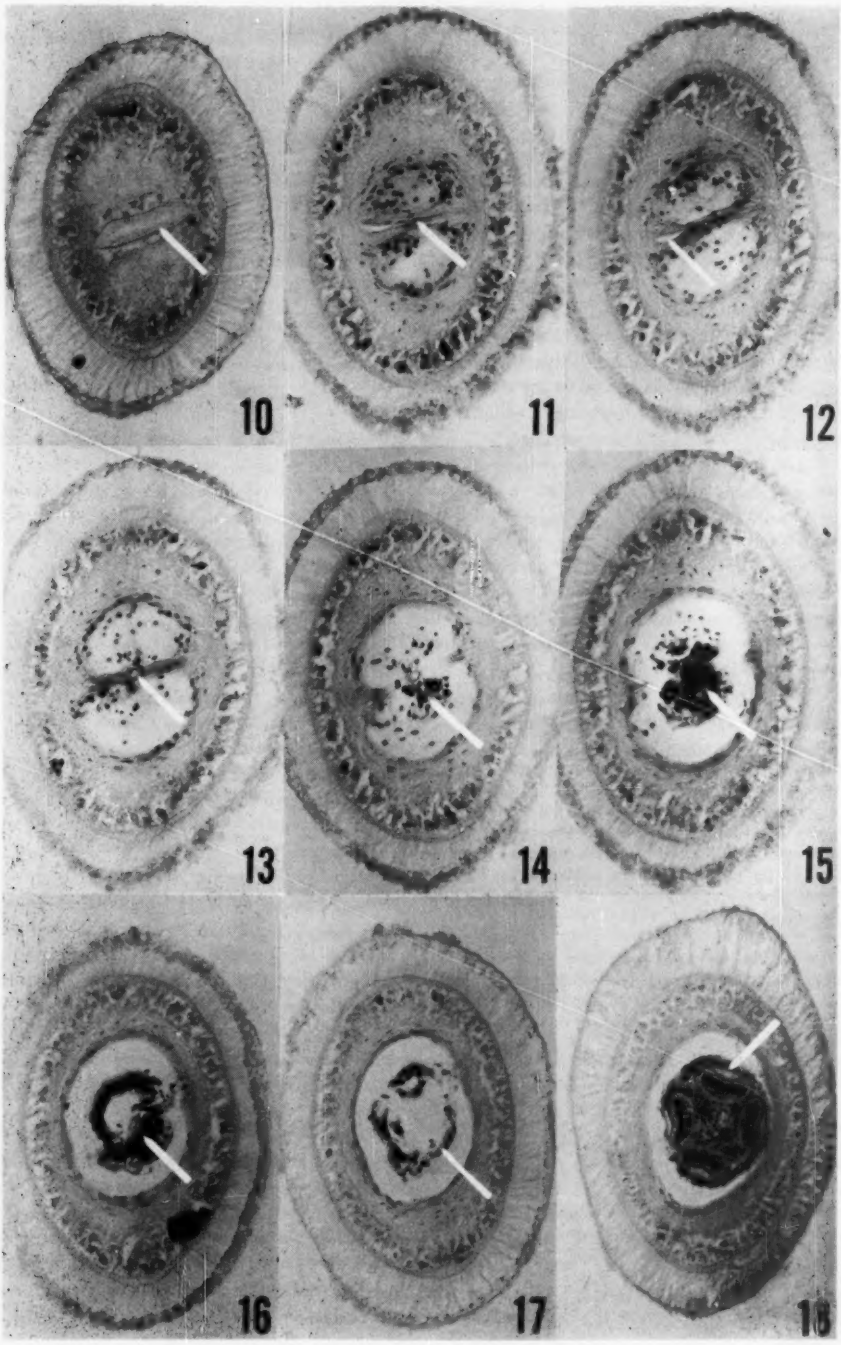


PLATE II



PROTEIN CONTENTS OF THE TAPEWORM *HYMENOLEPIS DIMINUTA* FROM NORMAL, BILELESS, AND STARVED RATS*

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In previous studies (Goodchild, 1958, 1960, 1961 in press), deleterious effects upon *Hymenolepis diminuta* maintained in bileless and starved Wistar strain rats have been reported. Depression in length, wet and dry weight, and in total carbohydrate and glycogen have been found to occur in tapeworms grown in starved rats and in rats whose small intestines have been deprived of a normal bile flow by surgically installed cannulae which rerouted bile to the caeca.

The present report is a continuation of this series and contains results of nitrogen determinations and protein calculations performed on tapeworms grown in normal, bileless, and starved hosts. Worms used were harvested for studies on wet weight, length, and place of attachment in surgically modified hosts (Goodchild, 1960, and for carbohydrate analyses (Goodchild, 1961 in press).

MATERIALS AND METHODS

Rats were each given, by stomach tube, one 18-day-old cysticercoid of *H. diminuta* grown in *Tribolium confusum*. On the 20th day an operation was performed on 25 infected rats to cannulate the bile duct to the caecum (Goodchild, 1958), 16 infected rats were starved until they were sacrificed, and 15 infected rats were maintained as controls on a standard diet of commercial rat food. All rats were sacrificed 27 days post-infection and tapeworms were recovered, weighed, homogenized, frozen in distilled water, and later freeze-dried and dry weighed. Nine worms chosen at random from each group of rats were used for computing mean dry weights; nine freeze-dried worms from normal hosts, seven from bileless hosts, and six from starved hosts were used in nitrogen determinations and computations of protein contents.

Nitrogen determinations were performed using a semi-micro Kjeldahl procedure essentially similar to that given by Kabat and Mayer (1948). Ammonia from 1 to 20 mg of dried tapeworm material was distilled into 2 percent boric acid solution. Titration of the ammonium borate with HCl to pH 4.6 was accomplished with the aid of a single glass and reference probe-type electrode with the Beckman Model G pH meter. Percent protein was calculated by multiplying percent nitrogen by 6.25.

All data were analyzed statistically using "Student's" t-test for probability of significance of difference between means.

RESULTS

Percentages of nitrogen and protein, based on dried weights, are given in table I. Comparisons between the three populations reveal mean nitrogen and protein values to be significantly different, with "p" values at less than the 2 percent level.

Table I also contains a column giving the sum of percent protein and percent carbohydrate (Goodchild, 1961 in press). As will be noted, the 60.0 ± 3.7 percent for worms from bileless hosts is significantly different from the composite sum in worms from normal hosts (69.9 ± 1.9). Worms from starved hosts contained 66.8 ± 2.6 percent protein and total carbohydrate, which is not significantly different from the composite sum in either bileless or normal worms.

In absolute amounts of protein, worms from normal hosts contain 94.4 mg, worms from bileless hosts contain 17.8 mg (18.9 percent of amount in normal worms), and worms from starved hosts contain 42.8 mg (45.3 percent of amount in normal worms). Thus, worms from starved hosts contain 2.4 times as much protein as do worms from bileless hosts, although starved worms are only 1.7 times as heavy.

DISCUSSION

In a previous paper (Goodchild, 1961 in press) it was reported that total carbohydrate and glycogen were greatly depressed in worms from bileless and starved hosts as compared to those from normal hosts. Absolute amounts of protein were similarly depressed in worms from experimental hosts as compared to those from

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TABLE I. *Comparison of percent nitrogen and protein and percent protein plus percent total carbohydrate in* Hymenolepis diminuta *from normal, bileless, and starved rats. Figures in parentheses represent numbers of worms analyzed.*

Host	Pct. nitrogen		Pct. protein ($N \times 6.25$)		Pct. protein + pct. total CHO		Dry weight (in mg)	
	Mean ± S.E.M	p values	Mean ± S.E.M	p values	Mean ± S.E.M	p values	Mean ± S.E.M	p values
Normal	5.12 ± 0.23 (9)		32.0 ± 4.4 (9)		69.9 ± 1.9 (9)		295.0 ± 13.1 (9)	
		< 0.02*		< 0.02*		< 0.05*		< 0.01*
Bileless	6.62 ± 0.55 (7)		41.4 ± 3.4 (7)		60.0 ± 3.7 (7)		43.0 ± 4.1 (7)	
		< 0.01**		< 0.01**		> 0.10**		> 0.05**
Starved	9.35 ± 0.43 (6)		59.5 ± 2.7 (6)		66.8 ± 2.6 (6)		72.0 ± 14.4 (6)	
		< 0.01***		< 0.01***		> 0.10***		< 0.01***

Probability of significant difference between :
* Worms from normal and bileless rats ;
** Worms from bileless and starved rats ;
*** Worms from normal and starved rats.

control hosts. The most interesting finding in these comparisons is that starved worms, although heavier than bileless ones, contain relatively less carbohydrate, but more protein.

Percentages of nitrogen (5.12 ± 0.23) and protein 32.0 ± 4.4) in dried, normal *H. diminuta* lie within, or close to, the ranges reported by other workers for other cestodes. Von Brand (1952) summarized data from the literature and gave values which ranged from 30 to 60 percent. Campbell (1960) reported only 21.85 percent protein in dried *Moniezia expansa* and stated that higher values given by others have been computed erroneously from totals which included non-protein nitrogen along with protein carboxyl nitrogen which probably represents the true amino acid content of cestode proteins.

The sum of total carbohydrate, protein and lipid (unpublished) does not equal 100 percent of the dried weight. There is a difference of about 10 percent for normal worms, 22 percent for bileless worms, and 6 percent for starved worms. Presumably this difference represents the inorganic components. It would be interesting to note the changes in calcium, especially in the bileless ones. Von Brand et al (1960) have stated that their studies ". . . lend some support to the view that one function of the calcareous corpuscles is to buffer anaerobically produced acids, but they fall short of providing definite proof." Obviously, a bileless environment differs in buffering potentialities from one containing bile.

A reciprocal percentage relationship between carbohydrates and proteins was expected in the experimental groups of worms. As carbohydrate percentages decreased some other organic or inorganic fraction should have increased. Proteins did increase in bileless and starved worms, but not at equivalent rates. In normal worms proteins were 84.4 percent of total carbohydrates, in bileless worms they were 222.6 percent of total carbohydrates, and in starved worms they were 815.1 percent of total carbohydrates. Relative to an assumed unity ratio of protein to carbohydrate in normal worms, bileless ones have a ratio of 2.7 and starved worms a ratio of 9.7.

SUMMARY

Kjeldahl nitrogen determinations and calculations for protein contents have been made on 27-day-old freeze-dried *Hymenolepis diminuta*, the rat tapeworm, recovered from Wistar strain rats and on worms maintained for the last 7 of the 27 days in bileless and starved rats. Mean nitrogen percentage values for nine normal worms was 5.12 ± 0.23, for seven bileless worms was 6.2 ± 0.55, and for six starved worms was 9.35 ± 0.43. Computed protein percentages ($N \times 6.25$) were 32.0 ± 4.4 for normal worms, 41.4 ± 3.4 for bileless worms, and 59.5 ± 2.7 for starved worms. When mean dry weights were used to compute absolute amounts of protein it was calculated that normal worms contained 94.4 mg, bileless worms 17.8 mg, and starved worms 42.8 mg.

Worms from normal hosts contain only about 85 percent as much protein as total carbohydrate. On the other hand, relatively

much more protein than total carbohydrate was found in bileless worms (222.6 percent) and in starved worms (815.1 percent).

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RESEARCH NOTE

POMATIOPSIS LAPIDARIA SAY (GASTROPODA: AMNICOLIDAE) IN LOUISIANA.

Pomatiopsis lapidaria is the common first intermediate host of the American lung-fluke, *Paragonimus kellicotti* Ward, 1908 (Ameel, 1934, Am. J. Hyg. **19**: 279–317). Berry and Rue (1948, J. Parasit. **34** (supp.): 15) have also indicated that *P. lapidaria* may experimentally serve as the first intermediate host for *Schistosoma japonicum* Katsurada, 1904, the Oriental human blood fluke.

Ameel (1934) and La Rue and Ameel (1937, J. Parasit. **23**: 382–387) recorded the metacercariae of *Paragonimus* from an unidentified crayfish in Oberlin, Louisiana. The latter authors suggested that other snails, especially amnicolids, may serve as first intermediate hosts of the American *Paragonimus*. Abbott (1948, Proc. U. S. Natl. Mus. **98**: 57–67) discussed the American distribution of *P. lapidaria* and noted the lack of any record of its presence in Louisiana. He suggested that the presence of *P. lapidaria* should be suspected wherever the American *Paragonimus* is found. Basch (1959, J. Parasit. **45**: 273) infected the amnicolids *Oncomelania nosophora* (Robson) from Japan and *Pomatiopsis cincinnatiensis* (Lea) from Michigan, with *Paragonimus kellicotti*. He also exposed *Oncomelania formosana* (Pilsbry and Hirase) from Formosa and *O. quadrasi* (Moellendorf) from the Philippines to miracidia of *P. kellicotti*, but was unable to infect these species.

In view of La Rue's and Ameel's suggestion that the American *Paragonimus* may develop in amnicolids other than *P. lapidaria*, and of Basch's supporting evidence, we felt that the subject should be investigated further, especially because of Abbott's comments regarding the overlap of the distribution of *Paragonimus kellicotti* and *P. lapidaria*.

We were informed by Drs. Paul C. Beaver and H. Harry of one unpublished record of *P. lapidaria* in Louisiana by Harry (1942, M. S. Thesis, Louisiana State University) who in a footnote reported the species from ". . . Comite and Amite Rivers at their junctions."

The present report of *P. lapidaria* is from the west bank, on the north side of the U. S. Highway 190 bridge, Amite River, Louisiana. The Amite River bounds the study area on the east side, and a high road embankment is on the south and northeast sides. The habitat is a flood plain which has a stand of maple trees. The snails were found on elevated areas, within the flood plain, where the drainage and seepage from the higher adjacent land has eroded the sandy loam soil leaving high spots which frequently resemble small islands.

The snails were mainly found in humid spots between maple leaves, rarely crawling on the upper surface of the leaves. During the middle of December the air temperature was 6 C and the microhabitat temperature ranged from 8 to 9 C, depending upon the amount of leaves covering the snails. Absence of eggs and newly hatched snails was noted at that time of the year and until early February 1961 breeding had not started. The size of the snails collected in December suggests that some hatched the previous spring and others in the summer. Ecological information is being gathered and compared with similar information on this species of snail in the northeastern United States (van der Schalie and co-workers at Michigan).

Fifty *P. lapidaria*, some of them actively moving about at 8 C temperature, were collected from the Amite River locality and when isolated in the laboratory none shed cercariae. This study supported in part by research grants (E-3386 and E-2898) from the National Institutes of Health.—FRANKLIN SOGANDARES-BERNAL AND EMILE ABDEL-MALEK, *Departments of Zoology and Tropical Medicine and Public Health, Tulane University, New Orleans, Louisiana.*

STUDIES ON THE BIOLOGY OF *HYMENOLEPIS MICROSTOMA* (DUJARDIN, 1845)*

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In the fall of 1959 this laboratory began rearing *Hymenolepis microstoma* Dujardin (1845) recovered from mice of two areas near Chattanooga, Tennessee. As we were studying the effects of radiation on host-parasite relations in *Hymenolepis nana* (von Siebold, 1852) and *Hymenolepis diminuta* (Rudolphi, 1819), we felt that *H. microstoma* would be useful both to corroborate existing data and (because of its unusual site in the definitive host) to provide a research tool for future work.

Joyeux and Kobozieff (1927, 1928) described both the biology of the cestode and the distribution of the parasite. The following summary was composed from their paper of 1928, showing the distribution of *H. microstoma* as known at that time, and five more recent references:

LOCALITY	AUTHOR	FORM
Rennes (France), 1845	Dujardin	adult
Lille (France), 1880	Moniez	larva
Neighborhood of Froyes (France), 1928	Joyeux & Kobozieff	adult & larva
Niemegk, Potsdam area (Germany), 1853	Stein	larva
Göttingen (Germany), 1896	V. Linstow	larva
Zoölogical Museum of Berlin, 1906	Janicki	adult
Catane (Sicily), 1892	Grassi *et al.*	larva
Beni-Ounif (North Africa), 1928	Joyeux & Kobozieff	adult
Ireland	Diesing, 1851 (*fide* Wardle & McLeod, 1954)	adult
Rangoon (Burma)	Meggitt & Subramanian (1927)	adult
Argentina (S. America)	Bacigalupo (1928)	adult
Tanganyika (Africa)	Baylis (1934)	adult
North Carolina (U.S.A.)	Harkema (1946, 1949)	adult
Tennessee (U.S.A.)	Present study (1959)	adult

The question of synonymy of *Hymenolepis microstoma* with *H. muris-sylvatici* (Rudolphi, 1819) and *H. crassa* (Janicki, 1904) was discussed briefly by Hughes (1940), who cited Baer (1930). The latter differentiates between *Hymenolepis crassa* and *H. microstoma* on the basis of rostellar hooks, which in *H. crassa* have the handle much longer than the blade. *H. muris-sylvatici* has hooks of the same general type, unlike the hooks of *H. microstoma*. Since in our opinion the latter cestode will become widely used, we have here attempted to clarify its taxonomic status.

OBSERVATIONS

The Adult

Taxonomic diagnosis (Joyeux and Kobozieff, 1928): Length 80 to 350 mm; width 2.0 mm (maximum); holdfast (width) 200 microns; rostellum 100 microns; hook number 27; hook length 15.0 microns; testes (in line) 170 by 120 microns; cirrus pouch 135 by 50 microns; egg 80 to 90 microns; oncosphere 30 microns; hexacanth hook 17 to 20 microns; ovary (present study) 300 by 55 microns.

Our specimens agree essentially in these measurements except for the testes, which in the strain we are currently using measure 70 to 130 microns as compared with the 170 microns found by Joyeux in his strain. Also the rostellum bears 23 to 28 hooks in our strain as compared to the constant number of 27 found by Joyeux. The hook morphology was found to be basically the same as that described by Joyeux and Kobozieff (1928):

End of handle to point of blade, 15.0 microns.
End of guard to point of blade, 6.0 microns.
End of guard to end of handle, 14.5 to 15.0 microns.

The adult cestodes according to Joyeux and

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Kobozieff (1928) have a length of 80 to 350 mm, depending upon methods of relaxation and fixation. In the present study we found that the cestodes (after relaxation in 0.85 percent NaCl at 4 C and fixation in hot alcohol-formalin-acetic acid) vary in length in direct proportion not only to the age of the cestode, but also to the total number of cestodes harbored by the host. There is a positive crowding effect similar to that described by Read (1951), but considerably more pronounced than that described for *H. nana* by Schiller (1959). This difference is undoubtedly due to the unusual location of the cestode in the definitive host (bile duct and biliary passages). In a single worm infection of 14 days duration the cestode measures over 100 mm in length. This length decreases proportionately with an increased worm burden. From a 100 worm infection of 14 days duration 93 worms were recovered measuring from 10 to 30 mm. From single worm infections of 21 days duration the longest worm was 280 mm. In a 10 worm infection of 21 days duration the worms average 150 mm in length. After maturing the worm sheds its terminal proglottids and from that time until it is passed from the host (an average of 6 months) it sheds varying lengths of its strobila. After 6 months, cestodes from singly infected hosts average 205 mm in length. Apparently the taxonomic value of length in this species is rather doubtful.

Eggs

The egg of *H. microstoma* measures 75 to 90 microns in length as compared to 40 to 50 microns in length found in *H. nana*, and bears a striking resemblance to the latter. The internal morphology is similar except for the number of polar filaments (3–5 filaments per pole in *H. microstoma* compared to the 5–6 found in *H. nana*). It is our opinion that this size difference of the egg combined with the occurrence of polar filaments furnishes a simple taxonomic basis for identification of *H. microstoma*.

Cysticercoid

Joyeux and Kobozieff (1928) described the occurrence of the cysticercoid stage of *H. microstoma* in *Tenebrio molitor*, the large grain beetle. In his review of the literature he stated that both the larval and adult stages of the insect had been found by other workers to be naturally infected, but he could infect only the adult beetles. He thought that the larva must become infected only rarely, under certain unknown biological conditions. We allowed 35 adult and 35 larvae of *T. molitor* to feed on infected material. A careful examination of these insects subsequently failed to reveal cysticercoids. Apparently *T. molitor* resists infection by our cestode.

Three other coleopterous insects, however, have proven to be excellent intermediate hosts. These are the confused flour beetle *Tribolium confusum*, the related beetle *T. castaneum*, and the sawtoothed grain beetle *Oryzaephilus surinamensis*. Each of these insects is a newly recorded intermediate host for *H. microstoma*. The beetles are easily infected by the method described by Voge and Heyneman (1957). Cysticercoids, probably infective after one week (at 30 C), survive all ecdyses of the larva and remain infective for the life of the beetle. It has been found that the average number of cysticercoids recovered from an infected larva is considerably higher than that recovered from an adult infected in the same manner. The development of the cysticercoid follows the general pattern described for *H. nana* by Voge and Heyneman (1957). The effects of crowding upon the cysticercoid are apparent. The cercomer (normally 3 to 4 mm in singly infected beetles) is very small in multiple infections. The ease of infecting three different coleopterous insects indicates that there is at least a partial lack of host specificity in the cysticercoid stage of *H. microstoma*.

Establishment in the Definitive Host

The establishment of the cestode in the definitive host occurs only by means of the cysticercoid; we have not been able to produce direct egg infection experimentally. The establishment begins with the ingestion of the cysticercoid and the action of gastric secretions upon it. Both HCl and pepsin are required for the partial dissolution of the surrounding membranes of the cysticercoid. The bile salts of the upper duodenum activate the larval worm which subsequently excysts. The newly excysted cestodes measure on the average 0.15 mm. For 24 hours there appears to be a random wandering of the worms in the upper 25 percent of the intestine with little if any increase in size. After 48 hours the cestodes have become considerably more localized and their length has

increased to an average of 0.30 mm. By 72 hours the worms have again doubled in length (average 0.67 mm) and within 96 hours they have begun to establish themselves within the bile duct. The cestodes found in the bile duct after 96 hours average 1.6 to 2.0 mm in length. Up to this time the localization of the cestodes in the duodenum has become progressively more pronounced. At 120 hours (except for the cestodes in the bile duct, which average 2.7 mm) the cestodes remaining in the duodenum become more diffuse in their distribution and show little increase in length. By 144 hours the average length of the cestodes in the bile duct is 3.9 mm. Apparently those cestodes which have not at 144 hours reached the bile duct do not become established.

Habitat in the Definitive Host

One of the most remarkable features of the cestode *H. microstoma* is its habitat, the bile duct and biliary passages. Only certain other cestodes to our knowledge, some thysanosomines from ruminants and some anoplocephalines (*Hepatotaenia* spp.) from kangaroos, have been found normally living in this location as adults (See Wardle and Mcleod, 1952). Joyeux and Kobozieff (1928) refer to *H. microstoma* as follows (translation ours):

"The scolices are attached in the duodenum or bile duct. In the duodenum, the usual point of attachment is above and below the opening of the bile duct, within a range of 5 to 7 cm. In the bile duct, the usual attachment place is near the opening, in the first 4 millimeters; but the attachment can be much higher up. The strobilas, from this point of attachment, can develop in two different ways. In most cases the strobila describes a simple ring and returns over itself, thus reaching the intestine. But, at times, the worm is restricted to the cystic or hepatic ducts. Sometimes the worms completely fill the gall bladder. The latter is hypertrophied, embedded in the hepatic tissue. Its contents show fragments of degenerating worms. Found there were some quite young segments, some mature, and some gravid. If the worm enters the hepatic duct, it can go into the fine ramifications. The bile duct becomes enormously distended, equalling in diameter the duodenum, which is also hypertrophied."

We find this description to be essentially similar to our observations except for the relative frequency of attachment of the cestode in the duodenum. Of the hundreds of infected mice examined we have found only two instances of attachment in the duodenum. In all other cases the cestodes have been attached in the bile duct or biliary passages, with the ends of the strobila in the lumen of the duodenum.

Definitive Hosts

The mouse *Mus musculus* proves to be a suitable definitive host for *H. microstoma*. There appears to be no age immunity to the cestode like that described by Larsh (1951) for *H. nana*. Autoreinfection as reported by Heyneman (1953) for *H. nana* does not occur in *H. microstoma*. In dual infections of *H. microstoma* and *H. nana* there appears to be no damaging effect of one cestode species upon the other. The two species can coexist within the same host, possibly because of their relative isolation from each other within the host.

There are numerous references in the literature pertaining to the ability of the rat to harbor *H. microstoma* (Harkema, 1946, 1949; Joyeux and Kobozieff 1928). We, however, have had no success in infecting the rat, *Rattus norvegicus*, with this cestode. A total of 10 rats of various ages and of both sexes were experimentally administered 10 to 100 cysticercoids. They were examined 14 to 36 days after infection. All failed to harbor *H. microstoma*. This result is contrary to Joyeux and Kobozieff's (1928) finding that rats could be experimentally infected without difficulty. Our failure was probably due to a strain difference in the cestode.

In an attempt to establish an infection of *H. microstoma* by egg, approximately 2,000 mature eggs per mouse were administered through a stomach tube (the large number of eggs takes into account the extremely low infection rate found for *H. nana* as reviewed by Larsh, 1943). None of the five mice thus treated harbored any cestodes when examined.

An attempt was also made to infect the golden hamster, *Mesocricetus auratus*, with *H. microstoma*. A total of 25 cysticercoids per hamster was administered through a stomach tube.

After 14 days (the time required for appearance of eggs in the fecal material of infected mice) one hamster was examined. Externally the hamster appeared emaciated and listless. The bile duct of the hamster was found to harbor seven cestodes. These cestodes were

one-third the length of those which would normally be found in a mouse infected for the same length of time. The hamster appeared to be badly jaundiced, a condition not found in mice even after a six-month infection.

Sixteen days after the initial infection the second hamster died. It was found to be severely emaciated and jaundiced. This hamster also harbored *H. microstoma*.

It appears that although the golden hamster, *M. auratus*, may harbor an infection of *H. microstoma* (a new host record), the infection rate is considerably lower (30 percent as against 70 to 80 percent) than that found in mice. It also appears that the cestode has an adverse physiological effect upon the hamster, a condition not found in mice.

Pathology of Infection

After establishment of the cestode there are definite pathological changes in the liver of the host. These changes of the liver, as described by Dobrovolskaïa-Zavadskaïa and Kobozieff (1927), include the appearance of white spots at the surface of the liver (necrofibrosis of hepatic parenchyma in the form of layers). In the places where this process is started, one distinguishes in the hepatic cells pale nuclei and sometimes in addition small vacuoles. In the more advanced layers, one sees homogeneous, acidophilic spans, without nuclei, but still maintaining the regularity of their contour and of their arrangement. Finally, infiltration by migratory cells, especially by polynucleocytes, appears with the disappearance of all hepatic structure. We have found that this condition can be initiated in the mouse within 120 hours after infection and is a common occurrence. The second condition described by Dobrovolskaïa-Zavadskaïa and Kobozieff (loc. cit.) was small white points, drawn out or confluent. Microscopic examination of such points revealed parasites in the liver tissue. This condition was probably attributable to the presence of *Cysticercus fasciolaris* which these authors found coexisting with *H. microstoma* although the authors made no attempt at interpreting it as such.

DISCUSSION

There are a number of interesting differences between the Chattanooga strain of *H. microstoma* which we are culturing and the strains described by other workers. The size of the testes is the major morphological difference found in the adult. The length of the adult (being directly related to several environmental factors) is of a questionable taxonomic value. All other morphological features of the adult thus far studied appear to agree essentially with those found by Joyeux and Kobozieff (1928) and are of value in making a proper identification. The mature egg is especially useful in this respect. The striking similarity between the egg of *H. microstoma* and *H. nana* combined with the size difference between the two is an excellent feature for a taxonomic diagnosis.

Suitable hosts for both the intermediate and definitive stages of the life cycle appear to be different, for our strain, from hosts previously reported. Our inability to infect *Tenebrio molitor* (the only reported intermediate host) along with our success in infecting both *Tribolium* spp. and *Oryzaephilus surinamensis* seems to indicate that (1) there is at least a partial lack of host specificity and (2) the strain we are using is refractory to the heretofore reported intermediate host. Undoubtedly there exist other insects that would prove to be adaptable to the life of this cestode.

Our strain of cestode appears also to differ from others in respect to the definitive host. The failure of our *H. microstoma* to become established in the rat indicates that there is a complete lack of adaptation for this host. Further investigation should be carried out with other possible definitive hosts to find out whether they are (1) refractory, like the rat, (2) compatible with the development of *H. microstoma* with non-fatal pathological changes, like the mouse or (3), compatible with establishment only with resulting fatal pathological changes, like the hamster.

The relationship of *H. microstoma* to *H. nana*, both phylogenetic and ecological, ought to be studied. Ecological problems are being investigated. Phylogenetic, or evolutionary, relationships may be clear enough to warrant discussion here.

Baer (1951) describes the evolution of cestodes as a series of parallel physiological and morphological adaptations coextensive with evolutionary changes in the definitive host. He calls this form of host specificity "phylogenetic," because in cestode taxonomy, groups of related parasites generally characterize groups of re-

lated hosts.

The cestodes of rodents include a number of species of the large genus *Hymenolepis* (see Hughes, 1940, 1941). *H. microstoma,* as pointed out above, resembles *H. nana* very closely in morphology and life cycle. These cestodes differ from each other, however, in physiology (habitat) and in the size of the egg and embryo.

Perhaps these two cestode species represent recent divergence from a common ancestor which resembled one of them more than the other. Evidence for such divergence may be sought in various features of the worms. Of morphological features, nothing novel can be observed in either. Geographically, both are widespread, although apparently *H. microstoma* is far less common than *H. nana.* Physiologically, both species show evidence of being recent. *H. nana* is able to infect its final host directly. Since this ability is unique among cestodes, it supports the view that *H. nana* is "modern." Also, *H. nana* shows variability, several "strains" being known (Wardle and McLeod, 1952, p 455). Against this evidence is the evidence of widespread suitability of many hosts to *H. nana,* with little if any harm to these hosts. *H. microstoma* is not auto-infective, and is in this respect typical or "old-fashioned." However, it has a narrower range of suitable hosts than *H. nana,* and in all observed definitive hosts it causes damage. Perhaps the profound extent of this damage is the best existing evidence that *H. microstoma* is a recent evolutionary offspring of a *nana*-like ancestor.

In infected animals there is high incidence of pathological changes in the liver. The pathological changes may be of a purely mechanical nature, such as varying degrees of stenosis of the infected ducts. There may be damage similar to that found in human cases of biliary cirrhosis resulting from prolonged biliary obstruction which causes tortuosity of the biliary channels, proliferation of the terminal bile ducts, atrophy and necrosis of the hepatic cells, and proliferation of connective tissue about the portal areas at the periphery of the lobules (Anderson, 1948). Retention cysts of the panereas resulting from blockage of the pancreatic ducts (Anderson, loc. cit.) may occur. In addition, there may be changes of a physiological nature, dependent on the host-parasite relationship itself.

Chandler (1943) advanced the idea that a tapeworm's food requirements may be satisfied by absorption of substances from the living mucous membranes of the host gut with which the worm lies in contact. The reasons for his conclusion are (1) the location of some adult tapeworms in places other than the lumen of the host's gut, (2) the marked host specificity displayed by some cestodes, and (3) the fact that larval forms of some cestodes absorb nutrition directly from the host tissues.

The most recent attempt at validation of Chandler's idea has been of a biochemical nature, studies on the transaminase activity and amino acid uptake of certain cestodes. Wertheim et al (1960) have found that the hymenolepidid cestodes with which they were working have a very limited capacity to carry out transaminations. In kinetic studies they found that these same cestodes have a marked capacity for absorption of a variety of amino acids from a surrounding medium. This primary indication of a rather simple dynamic system supports Chandler's hypothesis and may be of considerable importance in explaining the pathological effects of *H. microstoma* on its host.

As has been previously stated, an infection of *H. microstoma* is often accompanied by acute pathological liver changes in the host. Because the vertebrate liver is extremely sensitive to nutritional changes, it is possible that the cestode causes these changes by directly absorbing vital substances from the host. This idea is supported by the fact that the lumen of the bile duct and biliary passages inhabited by the cestode is able to supply little nutritionally to the cestode which must obtain its food from the walls. Such absorption may in some instances lead to a critical deficiency in the host.

Copeland and Salmon (1946) and Hartroft (1953) found that in rats a deficiency of certain amino acids (notably cystine and methionine) and of the ammonium salt choline can cause pathological liver changes varying from carcinoma to cirrhosis and necrosis. As these substances are important metabolites for both host and parasite, a competition for them might occur resulting in the pathological changes found in infected animals.

Thus it is felt that the occurrence of pathological effects in *H. microstoma* infections indirectly supports Chandler's hypothesis of absorption from the host when examined in light of recent data on host nutrition and cestode

metabolic activity.

SUMMARY

A strain of *Hymenolepis microstoma* (Dujardin, 1845) was studied as to its distribution and host-relationships.

The cestode was found in mice from Chattanooga, apparently for the first time in this host in North America. Laboratory infections were easily maintained using albino mice and grain beetles (*Tribolium* spp. and *Oryzaephilus surinamensis*). Rats were refractory to infection, as was the large grain beetle *Tenebrio molitor*, both having been reported as suitable hosts for another strain of this cestode. Hamsters could be infected, but suffered severe damage; the worms did not grow well in hamsters.

Experiments on crowding showed that the restricted space of the worm's habitat (the bile duct) probably enhances the stunting effects of heavy infections. Double infections (*H. microstoma* with *H. nana*) did not affect either worm.

Establishment in the bile duct occurs during the first 120 hours after ingestion of the cysticercoid.

Pronounced pathological damage in hamsters and observable effects in mice are interpreted as possibly due to worm-induced nutritional deficiencies. This interpretation is compatible with Chandler's hypothesis of direct transfer of nutrients from host tissue to cestode, and is also supported by recent studies in pathology and biochemistry.

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OBSERVATIONS ON DEVELOPMENT AND HIGH TEMPERATURE SENSITIVITY OF CYSTICERCOIDS OF *RAILLIETINA CESTICILLUS* AND *HYMENOLEPIS CITELLI* (CESTODA: CYCLOPHYLLIDEA)*

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Studies on the effects of high temperature stress upon development of cysticercoids of *Hymenolepis diminuta* (Voge, 1959, 1961b) have shown that scolex withdrawal as well as normal tissue differentiation and alignment can be inhibited if stress is applied during the sensitive period of development. The performance of comparable experiments with other cestode species seemed desirable to determine the presence or absence of a temperature-sensitive period and, if present, to establish whether or not it occurred at a stage of development equivalent to that in *H. diminuta*. Furthermore, it was of interest to study a species which normally has the adult stage in a bird host. It was assumed that there might be greater resistance to temperature stress in forms which as adults live in hosts with body temperature higher than those of mammals.

The species chosen for these studies were *Raillietina cesticillus* and *Hymenolepis citelli*. Results of observations are reported below.

MATERIALS AND METHODS

Insect hosts used were a Hawaiian strain of *Dermestes vulpinus* maintained in the laboratory on a ground meat diet, and *Tribolium confusum* kept in enriched flour at 30 C. For all experiments involving high temperature stress only *Tribolium* was used. *Dermestes vulpinus* served for stock maintenance and observations on cysticercoid development of *Raillietina cesticillus*. Beetles were infected with *Hymenolepis citelli* as previously reported for *H. diminuta* (Voge and Heyneman, 1957). Eggs were obtained from a ground squirrel (*Citellus beecheyi*) kept in the laboratory. Eggs of a Hawaiian strain of *Raillietina cesticillus* were obtained by macerating gravid proglottids passed by chickens. Beetles which had been starved for one week were allowed to feed on eggs for three and one-half hours and were then transferred to clean containers with food.

Observations on normal cysticercoid development were based on infected insects kept at 30 C. For temperature stress studies, beetles were transferred from 30 C to 38.5 C for two day periods at different stages of cysticercoid development. Beetles were dissected in saline solution after the total development is normally completed; cysticercoids were counted and observed for gross developmental abnormalities.

RESULTS AND DISCUSSION

At 30 C, *Raillietina cesticillus* develops in *Tribolium confusum* or in *Dermestes vulpinus* to the infective cysticercoid stage in 14 days. Examination of beetles, maintained at 30 C, at different days of cysticercoid development showed that elongated stages with bi-partite body division were present at 6 to 6.5 days of development, tripartite body division, a well developed scolex, rostellum and suckers at 7.5 days, and a withdrawn scolex at 9 days. At 13 to 14 days one observes the prominent external folds described previously (Voge, 1960). Results of exposure to 38.5 C at days 6 to 8, or 7 to 9 of development are shown in table I.

TABLE I. *Effect of high temperature stress upon scolex withdrawal of cysticercoids at different periods of development.*

Species	Days of exposure to 38.5 C	Withdrawn	Non-withdrawn
Raillietina cesticillus	6–8	7 (1.9 pct.)	369 (98.1 pct.)
Raillietina cesticillus	7–9	181 (76.3 pct.)	56 (23.7 pct.)
Hymenolepis citelli	3–5	2527 (99.5 pct.)	10 (0.5 pct.)
Hymenolepis citelli	4–6	974 (78.2 pct.)	272 (21.8 pct.)
Hymenolepis citelli	5–7	224 (22.4 pct.)	777 (77.6 pct.)

Sensitivity to temperature stress is most marked when exposure occurs on days 6 to 8, scolex withdrawal being inhibited in nearly all individuals. If temperature stress is applied 1 day

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later, at 7 days development, sensitivity to temperature is much less and three-fourths of all cysticercoids have withdrawn the scolex.

Two conclusions may be reached from the above observations. Cysticercoids of *Raillietina cesticillus* are at least as sensitive to high temperature stress as those of *H. diminuta*. Consequently, relatively high environmental temperature and tolerance during growth of the adult worm in the bird host is not associated with comparable tolerance of the larval stage. Secondly, the sensitive period of *R. cesticillus* coincides with maximal growth and development of the cysticercoid, as it does in *H. diminuta*. In both species, maximal effects of stress were noted when exposure to high temperature was initiated at an equivalent stage of development, namely at the time of elongation of the larva and the beginning of body divisions.

Results obtained with *Hymenolepis citelli* show that this species also possesses a sensitive period which coincides with maximal growth and development of the cysticercoid. At 30 C, cysticercoid development is accomplished in 11 days, as shown by histological studies (Voge, 1961a). The data presented in table I indicate that temperature stress on days 3 to 5 of development does not affect scolex withdrawal. In *Hymenolepis diminuta* days 3 to 5 are the most sensitive to high temperature stress (Voge, 1959), while in *H. citelli* days 5 to 7 represent the sensitive period. At 5 days of development, the cysticercoid of *H. citelli is* elongate, roughly pear-shaped or may have progressed to the beginnings of body divisions. In *H. diminuta* this stage is reached at 3 days of development. As observed previously, in *H. diminuta* and also in *H. citelli* development rates of all cysticercoids within one beetle are not the same. This is probably the reason for the lack of uniformity of the effect of high temperature upon scolex withdrawal in cysticercoids from a single beetle host. It is also possible that these two species are more resistant to temperature stress than is *Raillietina* in which scolex withdrawal can be inhibited in almost all cysticercoids.

From these observations one may conclude that (1) temperature—sensitive periods have been demonstrated in the larval development of three different cestode species, (2) these sensitive periods occur at comparable times of development coinciding with maximal growth and differentiation of the cysticercoids, and (3) tolerance to relatively high temperature during growth of the strobilated phase in the vertebrate host does not necessarily imply a comparable tolerance of the cysticercoid stage in the insect host.

It should be noted that experiments with high temperature stress serve to confirm or amplify observations on normal developmental patterns, since they help to establish the time of the sensitive period and thus determine rather precisely the stage at which maximal development and differentiation begin. Also, from the information on the three species studied, it would appear that maximal growth and differentiation of cysticercoids occur in the middle or close to the middle of the developmental period. In *H. diminuta* with a total developmental period of 8 days, maximal growth occurs on days 3 to 5. In *R. cesticillus*, which requires 14 days, comparable development occurs at days 6 to 8. *H. citelli* requires 11 days for completion of development; maximal growth apparently occurs on days 5 to 7, which is again approximately in the middle of the total developmental period. Several more species should be studied before it can be concluded that this growth pattern is typical for cysticercoids.

SUMMARY

High temperature stress (38.5 C) applied during development of cysticercoids of *Hymenolepis citelli* and *Raillietina cesticillus* in the beetle *Tribolium confusum* showed the presence of a sensitive period coinciding in both species with the period of maximal growth and differentiation. Sensitivity, measured by failure of scolex withdrawal, was greater in *R. cesticillus* than in *H. citelli*. The results are considered with respect to the different vertebrate hosts and compared with those obtained previously with *H. diminuta*. In all three species, maximal growth and differentiation occur in the middle of the total period necessary for cysticercoid development.

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RESEARCH NOTE

ON THE DISTRIBUTION AND SIZE VARIATIONS OF *PHYLLODISTOMUM BUFONIS*.

Sixty Western Toads, *Bufo boreas* Baird and Girard, were collected from the vicinity of the University of Colorado Summer Camp 3 miles southwest of Ward, Colorado, located on the eastern slope of the Rocky Mountains. These toads were infected with the bladder fluke, *Phyllodistomum bufonis* Frandsen, 1957. However, leopard frogs, *Rana pipiens* Schreiber, collected from the same area, as well as red-spotted toads, *Bufo punctatus* Baird and Girard, Woodhouse's toads, *Bufo Woodhousii* Girard, three-lined tree frogs *Psuedacris nigrata* Wied, and axolotls, *Ambystoma tigrinum* Green, collected from various locations in Boulder County, Colorado, were not found to be infected with this fluke.

Crawford (1939, J. Parasit. (Supp.) **25**: 26; 1940, J. Parasit (Supp.) **26**: 38) reported a *Phyllodistomum* parasite presumably of the species *P. americanum* Osborn taken from the Western Toad collected at the Rocky Mountain Biological Station on the western slope of the Colorado Rocky Mountains. Upon examination of 18 of the specimens which he collected, it is apparent that these parasites are morphologically similar to *P. bufonis* and should be considered as this species rather than *P. americanum*. This designation is based on host specificity, since *P. americanum* has been reported only in salamanders, as well as on morphology.

To show the extremes in morphological variations among the specimens collected from the University of Colorado Camp, 26 out of 927 parasites were selected and measured. A summary of these measurements, those of Frandsen (1957, Tr. Am. Micr. Soc. **76**: 329–332), as well as measurements of 10 specimens of the parasite collected by Crawford, are shown in table I. It

TABLE I. *A comparison of size between* Phyllodistomum bufonis *collected from three locations.*

Structure	Utah	Eastern slope, Colorado	Western slope, Colorado
Width of body	2.0–2.74 (2.07)*	0.8–2.6 (1.8)	0.7–1.2 (1.0)
Length of body	7.5–9.0 (8.0)	3.6–6.4 (4.8)	3.0–4.1 (3.4)
Oral sucker diameter	0.42–0.55 (0.47)	0.32–0.62 (0.46)	0.32–0.38 (0.34)
Acetabulum diameter	0.87–1.0 (0.92)	0.45–1.07 (0.71)	0.53–0.64 (0.59)
Ratio of oral sucker to acetabulum	1 : 1.73–1 : 20 (1 : 1.95)	1 : 1.09–1 : 1.84 (1 : 1.56)	1 : 1.40–1 : 1.87 (1 : 1.72)
Length of ovary	0.63	0.36–0.51 (0.40)	0.19–0.36 (0.29)
Width of ovary	0.43	0.19–0.40 (0.30)	0.11–0.19 (0.17)
Length of anterior testis	1.00–1.37 (1.23)	0.55–0.94 (0.74)	0.30–0.44** (0.36)
Width of anterior testis	0.40–0.82 (0.54)	0.26–0.79 (0.49)	0.17–0.28** (0.23)
Length of posterior testis	1.15–1.75 (1.48)	0.58–1.25 (0.91)	0.42–0.63** (0.63)
Width of posterior testis	0.40–0.80 (0.56)	0.30–0.75 (0.50)	0.15–0.28** (0.22)

* Average of specimens measured.
** Badly distorted.

is apparent from these measurements that *P. bufonis* differs greatly in size. In addition to the differences in measurements, the following anatomical features were noted: (1) In the specimens collected by Frandsen, the testes were tandem whereas in those collected in Colorado they varied from oblique to tandem; (2) the specimens collected in Utah had vitelline glands slightly less than one-half the size of the ovary, while the Colorado ones had a variation in size of from less than one-half to three-fourths the size of the ovary. In other respects the flukes appear to be similar and all should be considered as members of the species *P. bufonis*.

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RESEARCH NOTE

AN ATTEMPT TO INFECT MOLLUSKS WITH *ACANTHAMOEBA* SP.

Culbertson et al 1959, (Am. J. Path. 35: 185–197) found that *Acanthamoeba* sp., when introduced into mammals (mice and monkeys), attacks mucoid tissue. Although this protozoan was studied in its relation to mammalian hosts, it seemed desirable to determine whether it would infect other groups of animals. Since mollusks have large amounts of mucoid tissue, an attempt was made to infect some of them.

Infections were attempted with three species of mollusks: an aquatic pulmonate, *Biomphalaria sudanica;* an amphibious operculate, *Oncomelania nosophora;* and a terrestrial pulmonate, the slug *Arion circumscriptus.* The cultures of *Acanthamoeba* sp. (Culbertson et al, op. cit.) used in these tests were obtained from Eli Lilly and Company.

Arion circumscriptus. Four different methods of infection were attempted, each involving the use of five adult individuals (maintained on wet filter paper in nine-centimeter petri dishes): (1) 0.05 ml. of the culture was injected through the foot into the coelom, (2) two drops of the culture were placed on the back of each animal (over the opening of the respiratory pore), (3) twenty-five drops of the culture were placed on the filter paper so that the mucoid tissue of the foot would come into contact with the organisms, and (4) the slugs were submerged in a small dish of culture for 5 minutes (the animals were active so that all parts of the body, including the respiratory pore, were exposed to the organisms).

Controls for each of these four experiments were subjected to the same procedure as above, but only the uninfected culture medium (proteose peptone, skimmed milk, yeast extract, and phytone) was utilized. Five slugs, not treated in any manner, were maintained in a petri dish as controls for the entire experiment.

In all four tests both the experimental and control animals died within a day of each other. The length of survival (in days) of both controls and experimental animals in each of the four tests follow: (1) 14 to 15, (2) 6, (3) 3, and (4) 18. Controls for the entire study were still alive at the end of 24 days.

Biomphalaria sudanica. Twenty-five individuals (all ages, from newly hatched to adult) were placed in a dish of *Acanthamoeba* culture for 5 minutes. The animals became active immediately after being placed in the dish so that they were thoroughly exposed to the organisms. They were then placed in an aquarium. All were alive at the end of 30 days.

Oncomelania nosophora. Twenty-five adult snails were placed in a dish of *Acanthamoeba* culture for 5 minutes. As with the other two species, the animals became active and were thoroughly exposed to the organisms. These snails were maintained in a 3-inch clay saucer filled with mud. All were still alive at the end of 14 days. At this time approximately 3 milliliters of *Acanthamoema* culture were poured over the mud surface of the dish. All animals were still alive 16 days later when the experiment was terminated.

To determine if the *Acanthamoeba* actually invaded the tissues, the attempted infections were repeated (5 individuals of *Arion* and 25 each of *Biomphalaria* and *Oncomelania* were immersed in the culture for 5 minutes and maintained as above). At the end of 5 days all the *Arion* and seven individuals each of the other two species were removed, relaxed with nembutal, fixed with Bouin's fluid, and sectioned. The tissues were stained with hematoxylin and eosin and examined microscopically for indications of tissue damage or the presence of *Acanthamoeba* (tissues of unexposed individuals were examined for comparison). Particular attention was paid to areas rich in mucoid tissue. No indication of either tissue damage or the presence of the *Acanthamoeba* was observed in any of the specimens.

Discussion. In these experiments the mucoid tissues of the mollusks were thoroughly exposed to *Acanthamoeba,* particularly those individuals that were submerged in the culture medium. Not only were the organisms in contact with the foot, which is rich in mucoid tissue, but also reached the respiratory system. Although the culture medium itself appeared toxic to *Arion circumscriptus,* there was no evidence of mortality or infection from the exposure to the *Acanthamoeba* in any of the three species of mollusks tested. Each species belongs to one of three major groups of Gastropods, representing aquatic, amphibious and terrestrial forms. It is apparent, therefore, that *Acanthamoeba* will not infect Gastropod mollusks.

I wish to thank Drs. Culbertson and McCowen of Eli Lilly and Company for suggesting the problem and supplying the cultures of *Acanthamoeba.* Appreciation is also expressed to Mrs. Annette van der Schalie for preparing the sectioned material.—LOWELL L. GETZ, *Museum of Zoology, University of Michigan, Ann Arbor.*

PARASITOLOGIC SURVEYS IN CALI, DEPARTMENTO DEL VALLE, COLOMBIA. IX. ENDOPARASITES OF RODENTS AND COCKROACHES IN WARD SILOE, CALI, COLOMBIA

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This study was carried out during 1958–1959 in connection with epidemiologic investigations of human parasitosis in Ward Siloé, Cali (Faust, 1958, 1959; Faust, Giraldo, Caicedo and Bonfante, 1961; Faust and Read, 1959; Giraldo, Faust, Bonfante and Caicedo, 1959; Krupp and Faust, 1959; Faust and Giraldo, 1961). The rodents were trapped and the cockroaches collected in representative homes of the area. They were brought to our laboratory where they were sacrificed and examined for endoparasites.

METHODS

A study was made of 92 rodents (40 adult and 31 young *Rattus norvegicus*, 11 adult and 5 young *R. rattus rattus* and 5 adult *Mus musculus*). After the abdominal and thoracic viscera were exposed by a midventral incision, each rodent was inspected for macroscopic lesions and evidence of parasites. Then the entire digestive tract was removed, opened, and the contents transferred to suitable containers and slides. Direct unstained and iodine-stained mounted films of the fecal material were supplemented by zinc sulfate centrifugal flotation concentrates. Previous to sacrifice drops of blood had been obtained from each animal by cutting off the tip of the tail, for search in wet and stained dry mounts for hemoparasites. Our findings are presented in table I.

A total of 500 *Periplaneta americana* (242 adult and 258 immature) were examined. The viscera were exposed by an incision along the midventral line, the contents of the hemocoele were transferred to slides for wet-mount examination, then the intestinal tract was removed, opened and its contents placed on slides for study. The findings are summarized in table II.

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PRESENTATION OF DATA

Rodents. At least one endoparasite was discovered and diagnosed from 93.5 percent of the 92 rodents, including all of the adult rats and all of the mice. *Entamoeba histolytica* was found only in eight *R. norvegicus*, the common rodent in our area. *E. muris* was frequently obtained from all three murine species, as were *Chilomastix bettencourti*, *Trichomonas muris*, and *Giardia muris*. *Hexamita muris* was seen only once, in a direct fecal smear from an adult *R. norvegicus*. *Eimeria nieschulzi* was a common intestinal inhabitant of both species of rats and *E. falciformis* was diagnosed from all of the mice. Among the helminths, *Heligmosomum* spp. were often found in the intestine of both species of rats, while *Syphacia obvelata* and *Strongyloides ratti* were diagnosed only once and twice respectively in the Norway rat. *Hymenolepis diminuta* was the only intestinal cestode seem parasitizing all three rodent species. (Human infection with *H. nana* has been diagnosed only rarely during examination of more than 6000 specimens of human feces from persons in Ward Siloé during the past 4 years.) The most common intestinal helminth in all species of rodents was *Moniliformis moniliformis*. There were occasional findings of larval *Taenia taeniaformis* in the livers of all three host species. No evidence was obtained of parasitic involvement of the esophagus, stomach, lungs, pleural or peritoneal cavity of these rodents.

The blood of approximately one-third of the rats contained *Trypanosoma lewisi*, almost always in scant numbers.

The following parasites, whose cysts or eggs were found only in small numbers in zinc sulfate concentrates, are classified as contaminants: *Entamoeba coli*, *Isospora belli*, *Ascaris lumbricoides*, *Trichuris* sp. and *Ancylostoma* (or *Necator*). These parasite objects could easily have been ingested in human or dog's excreta com-

TABLE I. *Endoparasites of the family Rattidae, ward Siloé and vicinity, Cali, Colombia.*

			Rattus norvegicus		*Rattus rattus rattus*		*Mus musculus*
			Adults	Young	Adults	Young	Adults
Total no. examined		92	40 (43.5)	31 (33.7)	11 (11.9)	5 (5.4)	5 (5.4)
Total no. positive		86 (93.5)	40 (100.0)	26 (83.9)	11 (100.0)	4 (80.0)	5 (100.0)
INTESTINAL PROTOZOA							
Entamoeba histolytica	C	8 (8.7)	5 (12.5)	3 (9.6)			
Entamoeba muris	T, C	54 (58.7)	22 (55.0)	22 (70.9)	6 (54.5)	2 (40.0)	2(40.0)
Chilomastix bettencourti	T	73 (79.3)	36 (90.0)	19 (61.3)	10 (90.9)	4 (80.0)	4 (80.0)
Trichomonas muris	T	65 (70.2)	33 (82.5)	15 (48.4)	9 (81.8)	4 (80.0)	4 (80.0)
Giardia muris	T, C	31 (33.7)	18 (45.0)	8 (25.8)	3 (27.3)	1 (20.0)	1 (20.0)
Hexamita muris	T	1 (1.09)	1 (2.5)				
Eimeria nieschulzi	O	58 (63.0)	24 (60.0)	23 (74.2)	7 (63.6)	4 (80.0)	
Eimeria falciformis	O	5 (5.4)					5 (100.0)
INTESTINAL HELMINTHS							
Heligmosomum spp.	A, E	25 (27.2)	15 (37.5)	3 (9.6)	6 (54.6)	1 (20.0)	
Syphacia obvelata	A, E	1 (1.09)	1 (2.5)				
Strongyloides ratti	A, L	2 (2.18)	2 (5.0)				
Hymenolepis diminuta	A	11 (12.0)	3 (7.5)	1 (3.2)	2 (18.2)	1 (20.0)	4 (80.0)
Moniliformis moniliformis	A, J	49 (53.2)	32 (80.0)	7 (22.6)	7 (63.6)	2 (40.0)	1 (20.0)
HEPATIC INFECTION							
Strobilocercus of *Taenia taeniaeformis*	L	9 (9.8)	5 (12.5)	1 (3.2)	1 (9.1)	1 (20.0)	1 (20.0)
PERIPHERAL BLOOD							
Trypanosoma lewisi		33 (35.9)	14 (35.0)	14 (53.8)	4 (36.4)	1 (20.0)	
PROBABLE CONTAMINANTS							
Entamoeba coli	C	1 (1.09)	1 (2.5)				
Isospora belli	O	1 (1.09)		1 (3.2)			
Ascaris lumbricoides	E	3 (3.3)			2 (18.2)	1 (20.0)	
Trichuris sp.	E	3 (3.3)	3 (7.5)				
Hookworm (*Ancylostoma* or *Necator*)	E	3 (3.3)	3 (7.5)				

T, trophozoite ; C, cyst ; O, oocyst ; A, adult ; L, larva ; J, juvenile ; E, egg.
Percentage incidence of infection is shown in parenthesis following the number of infected individuals.

monly deposited on the ground in and around the homes (Giraldo, Faust, Bonfante and Caicedo, 1959).

Cockroaches. Endamoeba blattae and *Balantidium praenucleatum* were obtained from the intestinal lumen of a relatively high proportion of both adult and immature specimens, but the nematode *Leidynema appendiculatum* was the prevalent intestinal parasite. Embryonated eggs of *Moniliformis monilifomis* were discovered in the intestinal lumen of one adult roach. Other parasite species diagnosed, considered to be contaminants of the intestine of the roach, were the rodent flagellates *Trichomonas muris* and *Chilomastix bettencourti,* cysts of *E. histolytica, E. coli,* and *Endolimax nana,* oocysts of *Isospora belli,* and egg of *Ascaris lumbricoides* and *Trichuris.* In the hemocele of several cockroaches

TABLE II. *Endoparasites of the intestinal tract and hemocele of the cockroach* (Periplaneta americana) *in ward Siloé and vicinity, Cali, Colombia.*

			Adults	Young
Total number examined		500	242 (48.4)	258 (51.6)
Total number infected		203	125 (51.6)	78 (30.2)
INTESTINAL TRACT				
Endamoeba blattae	T, C	102 (20.4)	44 (18.2)	58 (22.5)
Balantidium praenucleatum	T	266 (53.2)	133 (54.9)	133 (51.6)
Leidynema appendiculatum	A, L, E	294 (58.8)	140 (57.8)	154 (59.7)
Moniliformis moniliformis	E	1 (0.2)	1 (0.4)	
HEMOCELE				
Gregarina blattarum		30 (6.0)	21 (8.6)	9 (3.5)
Moniliformis moniliformis, acanthor, acanthella, juvenile		203 (40.6)	155 (64.0)	48 (18.6)
INTESTINAL CONTAMINANTS				
Trichomonas muris	T	332 (66.4)	157 (64.9)	175 (67.8)
Chilomastix bettencourti	T, C	2 (0.4)	2 (0.8)	
Entamoeba histolytica	C	17 (3.4)	10 (4.1)	7 (2.7)
Entamoeba coli	C	43 (8.6)	24 (9.9)	19 (7.4)
Endolimax nana	C	2 (0.4)	2 (0.8)	
Isospora belli	O	2 (0.4)	2 (0.8)	
Ascaris lumbricoides	E	13 (2.6)	8 (3.3)	5 (1.9)
Trichuris sp.	E	25 (5.0)	9 (3.7)	16 (6.2)

T, trophozoite ; C, cyst ; A, adult ; L, larva, E, egg ; O, oocyst.
Percentage incidence of infection is shown in parenthesis following the number of infected individuals.

there were luxuriant colonies of *Gregarina blattarum* and many developing stages of *M. moniliformis.*

DISCUSSION

The domestic rats of Ward Siloé were investigated to discover if these rodents serve as reservoirs of intestinal parasites of man in this area. The one probability concerns *Hymenolepis diminuta,* which has been occasionally diagnosed from the human population. Even though the rat is reported to be an occasional natural host of *Entamoeba histolytica* (Lynch, 1915; Andrews, 1934; Tsuchiya and Rector, 1935; Atchley, 1936; Chang, 1945), cysts of this ameba were recovered from only 8 *R. norvegicus,* and no trophozoites of this ameba were found in the contents of the colon or scrapings of the mucosa. It is concluded from our finding of only cysts of this species, as well as of *E. coli,* oocysts of *Isospora belli* and the nematode eggs, that they probably represent chance contamination from ingestion of human or dog's feces.

Entamoeba muris and the intestinal flagellates which we found in the Siloé rodents have been previously reported as natural parasites of these hosts (Andrews, 1934; Tsuchiya and Rector 1935; Kudo, 1954). Our recovery of *Eimeria nieschulzi* exclusively in the rats and *E. falciformis* only in mice supports the studies of earlier workers (Wenyon, 1926; Moorhouse, 1938) that *E. nieschulzi* Dieben, 1924, does not parasitize mice and that *E. falciformis* (Eimer, 1870) is not adapted to the intestinal tract of *R. norvegicus* and *R. rattus rattus.*

When rats and mice in the Siloé area were found to be common definitive hosts of *Moniliformis monilifomis,* an attempt was made to discover the intermediate host of this acanthocephalan. The most likely one, based on previous life cycle studies (Grassi and Calandruccio, 1888; de Magalhães, 1898; Seurat, 1912), was the cockroach, the only representative of which in our study area is *Periplaneta americana.* More than 40 percent of the 500 roaches examined by us had developmental stages of this acanthocephalan in their hemocele. The presence of *Trichomonas muris* as an intestinal contaminant in two-thirds of the roaches provides additional evidence of the close ecological relationship of domestic rodents and *P. americana* in Siloé, while the relatively common occurrence of cysts of *E. histolytica* and *E. coli* in the intestine of these roaches supports the view of earlier investigators (Macfie, 1922; Téjera, 1926; Frye and Meleny, 1936) that roaches are potential mechanical vectors of these human protozoa.

The frequent presence of *Endamoeba blattae* and *Balantidium praenucleatum* in the intestine, and of *Gregarina blattarum* in the hemocele of *P. americana* furnishes information of a new geographical location for these natural parasites of the cockroach. Similarly, the common finding of the oxyuroid nematode *Leidynema appendiculatum* indicates that it is as well adapted to *P. americana* in our area as it is to *Blatta orientalis* in the United States (Dobrovolny and Ackert, 1934). Our failure to find larval stages of *Gongylonema, Protospirura, Physaloptera,* and *Oxyspirura* in the Siloé roaches is not unexpected, since the rats examined did not harbor the first two spiruroids and the definitive hosts of the latter two have not been found in Ward Siloé. We are surprised that some of the *P. americana* did not harbor gordiid worms, which are occasionally found in this cockroach in other wards of Cali.

SUMMARY

In connection with epidemiologic investigations of human intestinal parasites in Ward Siloé, Cali, Colombia, 71 *Rattus norvegicus,* 16 *R. rattus rattus* and 5 *Mus musculus* were studied for endoparasites. More than 90 percent of all rodents examined were infected with one or more natural inhabitants of these hosts. Commonly diagnosed were: *Entamoeba muris, Chilomastix bettencourti, Trichomonas muris, Giardia muris, Eimeria nieschulzi* and *Heligmosomum* spp. in the rats, *Eimeria falciformis* in the mice, and *Hymenolepsis diminuta* and *Moniliformis moniliformis* in all three murine species. *Hexamita muris* and *Syphacia obvelata* were obtained once each and *Strongyloides ratti* twice from *R. norvegicus.* Larvae of *Taenia taeniaeformis* were occasionally present in the livers of all three hosts species and *Trypanosoma lewisi* was often diagnosed in the blood of both species of rats. Considered as likely chance intestinal contaminants due to ingestion of human or dog's feces were the following: cysts of *E. histolytica* and *E. coli,* oocysts of *Isospora belli* (once), and eggs of *Ascaris lumbricoides, Trichuris* sp. and hookworm, all from zinc sulfate concentrates. *Hymenolepis diminuta*

was the only parasite for which the rodents appear to serve as reservoir of human parasites.

Examination was made of 500 *Periplaneta americana*, the only cockroach in Siloé. *Endamoeba blattae, Balantidium praenucleatum* and *Leidynema appendiculatum* were common intestinal parasites, and *Gregarina blattarum* and developing stages of *Moniliformis moniliformis* frequent inhabitants of the hemocele. Thus *P. americana* constitutes the one known natural intermediate host of *M. moniliformis* in our study area. The following species of intestinal parasites were classified as chance contaminants: *Trichomonas muris* and *Chilomastix bettencourti* of the rat (trophozoites only), *E. histolytica, E. coli* and *Endolimax nana* (cysts), *Isospora belli* (oocysts), and *Ascaris lumbricoides* and *Trichuris* (eggs). The cockroach is probably a potential mechanical disseminator of the diagnosed species which are natural parasites of man.

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